昭和 32 年 7 月 9 日 第三種郵便物認可
第 44 巻 第 9 号 昭和 32 年 9 月 25 日発行
(毎月 1 回 25 日発行)
The Journal of Biochemistry, published monthly by the Japanese Biochemical Society, Tokyo, Japan.
Vol. 44, No. 9, September 25, 1957

Vol. 44, No. 9 **September, 1957**

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

PUBLISHED MONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle	**Maruzen Co. Ltd.**	**Santo Vanasio**
Rutlant, Vt.	Tokyo	Köln
U.S.A.	Japan	Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the biochemical subject. It is published monthly, one volume per year.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost; additional copies can be prepared by the order on cost accounts. All orders for reprints should be clearly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts besides original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units and periodicals, will be sent immediately.

Subscription

Subscription Price. ¥3,600 (U. S. $10.00) per volume, postage inclusive. Back issues are available for sale, from volume 1 up-to-date at ¥3,600 (U. S. $10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co.*, Rutland, Vermont, U.S.A., *Santo Vanasio*, Köln, Germany, and *Maruzen Co. Ltd.*, Chuo-ku, Tokyo, Japan.

The Journal of Biochemistry, Vol. 44, No. 9, 1957

THE CONTRACTILE PROTEIN FROM TUBE-FEET OF A STARFISH

By KOSÇAK MARUYAMA and HIROYUKI MATSUMIYA

(*From the Biological Institute, College of General Education, University of Tokyo and Department of Chemistry, Faculty of Science, Hokkaido University*)

(Received for publication, February 18, 1957)

During the course of the comparative studies on the interaction of the contractile protein with adenosine triphosphate (ATP) (*1*) the authors had a chance to investigate the similar phenomenon in an acto-myosin-like protein extracted from tube-feet muscles of a starfish. Since no report has been published on the contractile protein of echinodermates, so far as we know, and some interesting properties were observed by the present authors, the results will be briefly described here.

MATERIAL AND METHODS

Material—The giant starfish, *Asterias amurensis* was collected from the sea-bottom near the Akkeshi Marine Biological Laboratory, Hokkaido. Tube-feet were cut out from the starfish body and washed twice with cold distilled water.

Preparation of Contractile Portein—About 200 g. of the tube-feet were suspended in 700 ml. of the Weber-Edsall solution by means of a waring blender and placed for 24 hours in the cold. The extract was purified by repeating three times the dilution procedure, as described before (*2*). Finally the protein was dissolved in 0.6 *M* KCl and insoluble matter was removed by centrigfuation.

Reagents—ATP which was purified by ion-exchange resin chromatography was kindly supplied from Mr. S. Kitagawa. Tris (hydroxymethyl)-aminomethane (Tris) was purchased from L. Light Co.

Adenosinetriphosphatase (ATPase) Tests—The ATPase reaction was carried out in 0.033 *M* Tris buffer, pH 7.0, at 20°. with occasional shaking. The total volume of the reaction mixture was 1.5 ml. The enzyme reaction was stopped by adding 0.5 ml. of 20 per cent trichloroacetic acid. 1.0 ml. of the deproteinized filtrate was analyzed for inorganic orthophosphate (P) by a micromodification of the Lohmann-Jendrássik method (*3*), using a Shimazu spectrophotometer.

Other Tests—The change of light-scattering intensity was measured as previously reported (*2*). Superprecipitation was observed by naked eyes. Viscosity was measured in Ostwald type viscometers.

RESULTS

Light-scattering Change with ATP—The purified contractile protein dissolved in 0.6 *M* KCl was considerably turbid and yellowish in color. Owing to the turbidity, the percentage change of the light scattering of the original sample, though diluted to a considerable extent, was small: on addition of ATP, some 5–10 per cent drop was observed in the intensity of scattered light. When the solution was clarified by filterating through a Seitz filter, a pronounced change by the addition of ATP was observed. As shown in Fig. 1, on addition of sufficient amount of ATP the maximum drop reached near 50 per cent. However, even in the

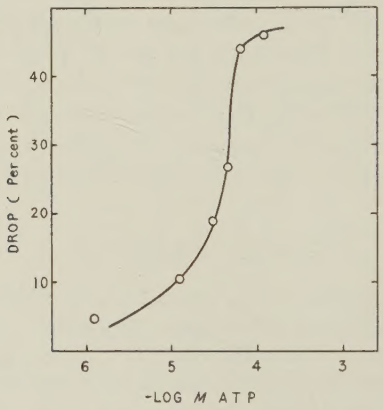


FIG. 1. Drop of the intensity of scattered light of the starfish contractile protein with ATP. pH 6.4 ; 20° ; 0.6 *M* KCl, 10 mM $MgCl_2$.

presence of 10 mM $MgCl_2$, the binding of ATP to the contractile protein might not so tightly as in rabbit (*2*) or pecten (*4*) myosin B. An S-shaped curve was obtained in plotting the increase of the percentage change against the ATP concentration added.

Superprecipitation with ATP—At the concentrations of 0.1–0.2 *M* KCl, the so-called superprecipitation (*5*) was clearly observed on addition of ATP at pH 6–7 at 20° (Fig. 2). Even in the presence of 0.6 *M* KCl, the concentrated protein solution showed some aggregation with ATP, which might show the difference in the solubility between this contractile protein and rabbit actomyosin. In fact the viscosity of such a sample was found to rise on addition of ATP. Perhaps more concen-

1. The failure to obtain a titration curve upon addition of ATP in the change of light-scattered intensity in the presence of 10 mM $MgCl_2$.

2. Although an appreciable drop in light-scattered intensity was observed in the dilute solution of the protein in 0.6 *M* KCl, in the concentrated solution some aggregation with ATP occurred under the same conditions. The latter fact suggests that the solubility of the starfish protein in KCl is less than that of actomyosins from vertebrate skeletal muscles (*cf. 5*).

3. In the presence of 0.6 *M* KCl, 10 mM $MgCl_2$ caused an appreciable increase in the ATPase action. This fact is of some interest that the contractile protein from a sea-anemone have the Mg-activated apyrase action (*8*), while the ATPase action of that from body-wall muscles of an annelid (*9*), from adductor muscles of pecten (*10*), from thoracic muscles of insects (*11*) and from vertebrate skeletal muscles (*2, 5*) was all inhibited by Mg under the similar test conditions. The enhancement of the ATPase activity by Mg ions in the presence of 0.1 *M* KCl as well as of 0.6 *M* may have some correlation with the fact that aggregation of the dense protein takes place with ATP in both cases (*cf. 6*).

It is possible that the low activity level of the ATPase action may be partly due to a contamination of an inert protein, such as tropomyosin A, recently found in invertebrate smooth muscles and characterized physicochemically by K. Laki's school (*12*).

SUMMARY

The contractile protein from starfish tube-foot muscles showed responses to ATP. It had an ATPase action, which was enhanced by Ca and to a less extent by Mg. ATP caused the so-called superprecipitation in 0.1 *M* KCl and drop in light-scattered intensity in the presence of 0.6 *M* KCl and 10 mM $MgCl_2$.

We wish to thank Assist. Prof. Y. Tonomura of Hokkaido University for his warm encouragements and also for his helpful criticism.

REFERENCES

(*1*) Maruyama, K., and Tonomura, Y., *J. Research Inst. for Catalysis* (*Hokkaido Univ.*) in press.

(*2*) Tonomura, Y., Watanabe, S., and Yagi, K., *J. Biochem.*, **40**, 27 (1953)

(*3*) Lohmann, K., and Jendrássik, L., *Biochem. Z.*, **178**, 419 (1926)

(*4*) Tonomura, Y., Yagi, K., and Matsumiya, H., *Arch. Biochem. Biophys.*, **64**, 456 (1956)

(5) Szent-Gyorgyi, A., Chemistry of Muscular Contraction. 2nd. ed. Academic Press. New York. (1951)
(6) Hasserbach, W., *Z. Naturforsch.*, **7b**, 163 (1952)
(7) Natori, R., and Shibuya, M., *Tokyo Jikei-kai Z.*, **68**, 931 (1952)
(8) Maruyama, K., *Biochim. et Biophys. Acta*, **16**, 589 (1955)
(9) Maruyama, K., *Enzymologia*, **17**, 90 (1954)
(10) Tonomura, Y., Yagi, K., and Matsumiya, H., *Arch. Biochem. Biophys.*, **59**, 76 (1955)
(11) Maruyama, K., *J. Fac. Sci., Univ. Tokyo*, **IV**, **7**, 231 (1954)
(12) Kominz, D. R., personal communication (1957) ; Also see Kominz, D. R., Saad, F., and Laki, K., *Nature*, **179**, 206 (1957)

The Journal of Biochemistry, Vol. 44, No. 9, 1957

ALCOHOLIC FERMENTATION BY INTACT CELLS OF BAKERS' YEAST

I. INHIBITION OF FERMENTATION BY PHOSPHATE

By MASAYA HAYASHIBE

(From the Research Department, Oriental Yeast Manufacturing Co. Ltd., Tokyo)

(Received for publication, March 14, 1957)

There are several articles dealing with the effect of phosphate on the fermentation of intact yeast cells (*1*, *2*, *3*), and it is generally accepted that extracellular phosphate does not take any definite part (*2*, *3*), notwithstanding the importance of phosphate in the mechanism of alcoholic fermentation. However, Trevelyan and Harrison (*4*) wrote from the result of their fermentation study that " phosphate caused a more rapid decline in later stages of glucose fermentation ". This phenomenon was also discovered during fermentation tests on industrial products of bakers' yeast, but under various fermentation conditions the phenomenon was not always observed.

After studying the nature of this phenomenon, the author has been able to determine definitely when it appears: namely, when the phosphorus-deficient or phosphate-starved yeast cells are placed in phosphate-containing sugar solution, metaphosphate accumulates within the cells accompanied with fermentation and perhaps deprives magnesium ion from the enzyme system, whereby the fermentation rate is greatly decreased.

METHODS

The Rate of Alcoholic Fermentation—Commercial bakers' yeast was washed with distilled water and prepared into a 10 per cent suspension (wet weight/volume). In a 100 ml. Erlenmeyer flask, 5 ml. of this yeast suspension, 5 ml. of 0.2 *M* citrate buffer solution (pH 5.0), and some phosphate *etc.*, were placed and the total volume was brought to 17 ml. with distilled water. This was well mixed and 3 ml. of sucrose solution (66.7 per cent (*w*/*v*) : granulated sugar of Dai-nippon Sugar Refining Co. Ltd.) was added. The final solution, with a total volume of 20 ml., became 10 per cent with respect to sucrose, 0.05 *M* per litre with respect to buffer solution, and 2.5 per cent with respect to commercial bakers' yeast.

The Erlenmeyer flasks were stoppered, fixed to the shaking apparatus in a ther-

mostat (30°), shaken for about 5 minutes, and then connected to a gas reservoir as is shown in Fig. 1. The gas produced was collected over a saturated sodium chloride solution and the volume was measured by the displacement of a salt solution into a measuring cylinder. The shaker was set for 110 oscillations per minute.

The fermentation was carried out for 3 hours with and without sodium dihydrogen phosphate (0.05 *M* per litre). The carbon dioxide evolution for each 30 minutes was observed.

The air in the flask was not displaced with nitrogen in these experiments, because the oxygen present was consumed in about 30 minutes, and thereafter fermentation was

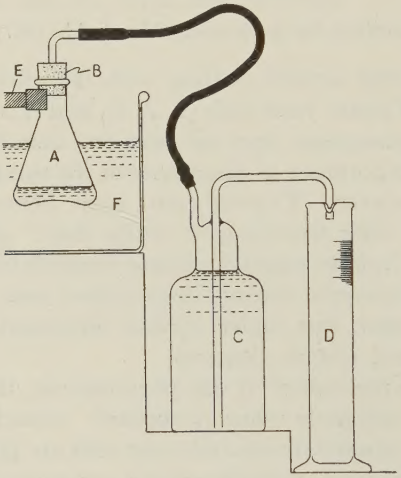


FIG. 1. Sketch of the fermentation apparatus.
A : Erlenmeyer flask (100 ml.) B : Rubber stopper
C : Gas reservoir (500 ml.), filled with saturated sodium chloride solution
D : Measuring cylinder E : Connected to shaker
F : Thermostat (30°)

carried out almost anaerobically. It was acertained by preliminary test that the fermentation rate of the above-mentioned system was almost equal under a strictly anaerobic condition except for the initial 30 minutes, and the effect of phosphate on the fermentation rate was essentially equal in both cases.

After 3 hours of primary fermentation, secondary fermentation with metal ions was carried out, if necessary.

Here the flasks were taken off the shaker and air was introduced to make the gas chamber conditions uniform, the metal solution to be tested was added, and again the above process was repeated.

The coincidence in the results of duplicate tests was always observed. An average of values from two experiments was plotted in the graph.

The Estimation and Fractionation of Phosphorus Compounds—Digestion of yeast cells and of extracts was carried out with perchloric acid. The inorganic phosphate formed was estimated by Fiske-Subbarow's method (*5*) except that perchloric acid was substituted for sulfuric acid. Extraction and partition of intracellular phosphorus compounds were carried out by the modified method of Schmidt and Thannhauser (*6*, *7*) ; cell materials to be tested were divided into three parts by successive extraction with cold trichloracetic acid (TCA) (at 0° to 4° ; 1 hour, 3 times) ; alcohol-ether (3:1) (at 80° ; 3 minutes, 3 times) ; and 1*N*NaOH (at 30° ; 5 minutes, 2 times). The orthophosphate in the TCA extract was precipitated by magnesia mixture, dissolved in a small amount of 1 *N* HCl, and estimated as above. The metaphosphate in the TCA and NaOH extract wss precipitated by barium acetate at pH 4.5 and the inorganic phosphate was estimated after 10 minutes hydrolysis with 1 *N* HCl (*7*).

Analysis was carried out before and after incubation with phosphate. After being fermented with the above mixture, it was centrifuged, washed, and returned to the original volume of a 10 per cent suspension, and analyzed parallel with the original.

Other experimental conditions are described under each individual experiment.

RESULTS

Inhibition of Fermentation by Phosphate—In a suitable buffer solution (citrate or phthalate), the fermentation rate of commercial bakers' yeast increased in the initial stage, but decreased in the late fermentation stage, in the presence of phosphate, contrary to the case in its absence (Fig. 2).

In a low pH range this decrease of fermentation rate by phosphate became mild as is shown in Fig. 3. When the phosphate was used as a buffer solution, the pH in the fermenting medium changed from pH 5.0 to about pH 3.5 in the first hour. Therefore, decrease in the fermentation rate by phosphate might be overlooked because of its mild effect in the low pH range.

When glucose was used instead of sucrose, the inhibition rate was the same.

It was also found that respiration was not affected by phosphate. After 3 hours of fermentation, the cells whose fermentation rate decreased were harvested, washed well, and again fermented as above. The rate of fermentation was still low, but in this case the phosphate did not cause further decrease in the fermentation rate (Fig. 4).

This phenomenon in question took place both anaerobically or aerobically. Therefore it may be considered that intracellular changes

caused by phosphate during fermentation or respiration inhibit some mechanism of fermentation.

Commercial bakers' yeast could be divided into two groups by the effect of phosphate on their fermentation. The one is inhibited by the presence of phosphate and the other is not effected by the phosphate. The phosphorus content of the yeast belonging to these two types differed significantly as indicated in Table I. The cells relatively poor in phosphorus content are affected by phosphate, and the phosphorus-rich cells are not affected by phosphate, although they will be affected by phosphate starvation (Fig. 5).

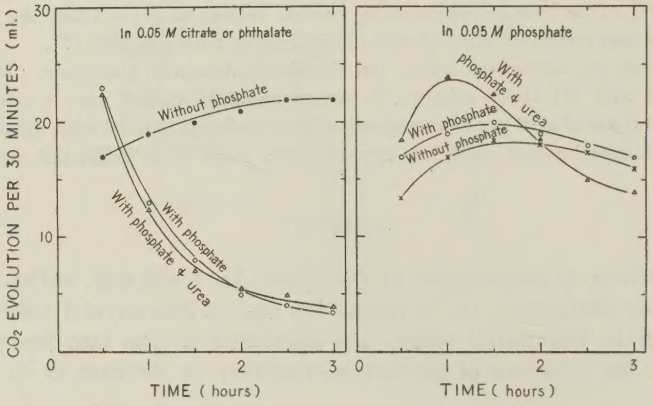


FIG. 2. Influence of phosphate on the fermentation rate in various buffer solutions. Temperature 30°, yeast 0.5 g., sucrose 2 g., buffer solution 0.05 *M*, phosphate 0.05 *M*, urea 0.09 g., initial pH 5.0, water to 20 ml. The final pH in the fermenting medium was 4.6 (citrate and phthalate buffer), 3.4 to 3.2 (phosphate buffer and without buffer), and 5.0 (phosphate buffer plus urea).

Intracellular Distribution of Phosphorus after Inhibition—As mentioned above, it is clear that the fermentation of phosphorus-poor cells is inhibited by extracellular phosphate. Many reports have appeared on the phosphate absorption by phosphate-starved cells during fermentation or respiration and its intracellular pattern (*8–14*). In the case of the phenomenon in question, the cells accepting inhibition absorbed much more phosphate than the cells not accepting inhibition, as indicated in Table II. The corresponding quantity of a greater part of the absorbed phosphate was found in the form of orthophosphate and meta-

phosphate (Table III).

Metal ions counteracted the inhibition, and magnesium ion especially released the cells from the inhibition even after they had been accepted inhibition. This will be explained in detail later. The cells incubated in the presence of phosphate with and without magnesium or calcium did not indicate any essential difference in the phosphorus pattern of the cells (Table IV).

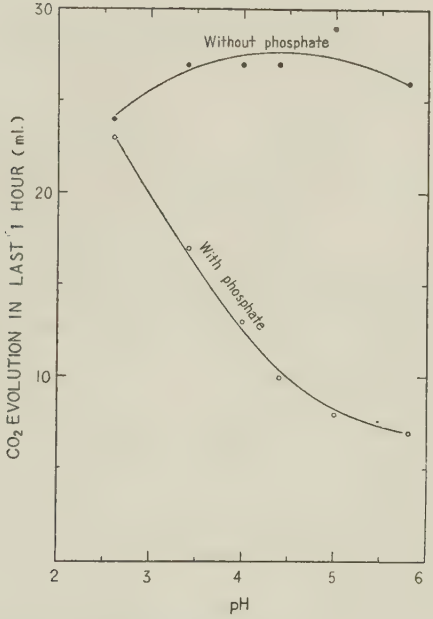


FIG. 3. Influence of pH on the fermentation rate with and without phosphate. Temperature 30°, yeast 0.5 g., sucrose 2 g., citrate buffer 0.05 *M*, water to 20 ml.

Inhibitors and Activators—Yoshida stated that the intracellular accumulation of metaphosphate was blocked by 2,4-dinitrophenol (DNP) and beryllium ion (*13*, *14*). Spiegelman *et al.* indicated that sodium azide inhibited the absorption of phosphate and the turnover of orthophosphate (*11*). In the present case, the phenomenon in question was not observed in the presence of 10^{-4} *M* DNP or NaN_3 (Fig. 6). This would indicate that the metaphosphate formation was closely related to

the fermentation inhibition by phosphate.

Effect of magnesium ion on the fermentation inhibition by phosphate (*4*) and on the metaphosphate accumulation of phosphate-starved cells

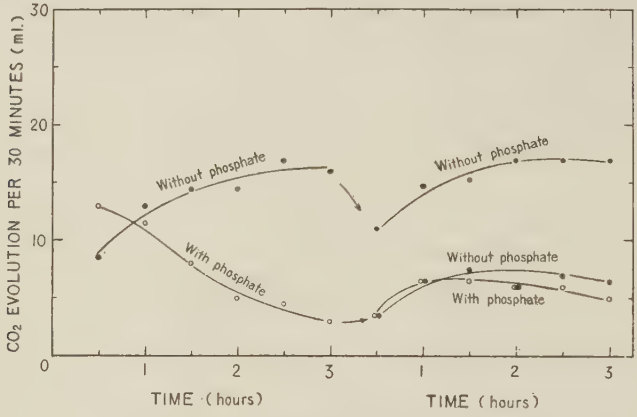


FIG. 4. Fermentation inhibition caused by phosphate within the cells. The yeast, fermented with and without phosphate, was washed with distilled water, and fermentation test was again carried out with corresponding quantity of yeast.

Temperature 30°, yeast 0.5 g. in primary fermentation, sucrose 2 g., citrate buffer 0.05 *M*, phosphate 0.05 *M*, water to 20 ml.

TABLE I

Fermentation Inhibition by Phosphate and Phosphorus Content of Cells

	Phosphorus (% on dry basis)	
	Cells, inhibitable by phosphate	Cells, not inhibitable by phosphate
No. of samples	16	16
Range of estimated values	1.26—0.60	1.49—1.26
Mean value with 95 per cent confidence limit	0.96±0.096	1.37±0.028

(*10*, *13*, *14*) have been reported. In the present case, when metal ions were added at the beginning of the primary fermentation, inhibition of fermentation did not occur in the case of magnesium, and inhibition was

partially recovered in the case of calcium or zinc (Fig. 7).

On the contrary, if metal ions were added at the beginning of the secondary fermentation, magnesium ion released the cells from the in-

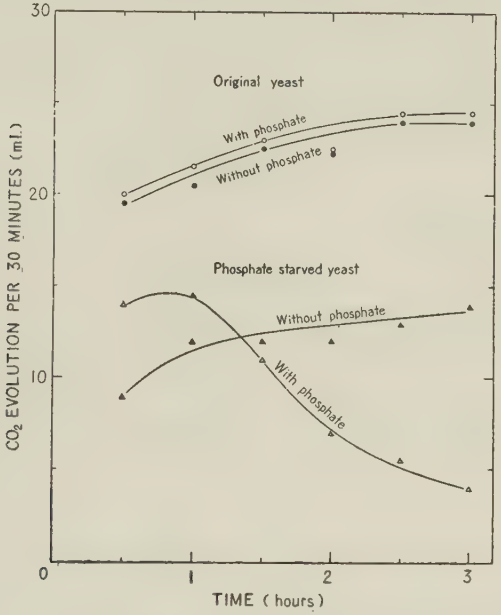


FIG. 5. Effect of phosphate on the fermentation rate of phosphate-starved yeast, whose fermentation rate was not affected by phosphate before starvation.

Temperature 30°, yeast 0.5 g., sucrose 2 g., citrate buffer 0.05 *M*, phosphate 0.05 *M*, water to 20 ml.

Phosphate starvation : 2 g., of yeast was shaken at 30° in 200 ml. of medium, containing glucose 2 g., urea 0.6 g., $MgSO_4 \cdot 7H_2O$ 0.05 g., KCl 0.02 g., sodium citrate 0.15 g., biotin 4 μg., calcium pantothenate 0.1 mg., pyridoxine 0.2 mg., thiamine 0.8 mg., inositol 2 mg., Fe^{++} 30 μg., Zn^{++} 80 μg., and Cu^{++} 5 μg.

After 16 hours of starvation yeast was harvested, washed, and prepared into a 10 per cent suspension. 5 ml. of this suspension was used for fermentation tests.

hibition but calcium or zinc ion did not indicate any kind of action (Fig. 8).

When fermentation was carried out without phosphate, magnesium

Table II

Absorption of Phosphorus during Fermentation

After 3 hours of fermentation, the cells were harvested, washed well with cold water, and the phosphorus content of the corresponding quantity of cells to the original cell quantity (Pai) was estimated. The phosphorus content of the original cells (Pbi) was also estimated at the same time, and the phosphorus absorbed per 100 mg. of the original dry cell matter (ΔP) was calculated.

		Cells, inhibitable by phosphate	Cells, not inhibitable by phosphate
No. of samples		7	7
ΔP (mg.)	Range	2.11–3.50	0.68–1.28
	Mean value with 95 per cent confidence limit	2.89±0.46	0.93±0.24
Pai/Pbi	Range	3.05–5.17	1.50–1.92
	Mean value with 95 per cent confidence limit	4.21±0.77	1.69±0.10

Table III

Intracellular Distribution of Absorbed Phosphorus after Fermentation with Phosphate

The yeast cells, inhibitable by phosphate, were fermented 3 hours with phosphate, harvested, washed with cold water, fractionated, and the phosphorus estimated. The phosphorus content of each fraction was compared with corresponding quantities of cell material before and after fermentation, and the difference per 100 mg. of the original dry cell material (ΔP) was calculated in each fraction.

Pt, Po, Pm, and Pmi signify, respectively, the total phosphorus of cells, the phosphorus of orthophosphate, of cold TCA- soluble and -insoluble metaphosphate.

Expt. No.	ΔP (mg.)				ΔP (% to total phosphorus increase)	
	Pt	Po	Pm	Pmi	Po+Pm+Pmi	Pm+Pmi
1	2.49	0.18	0.83	1.07	84.1	76.8
2	3.35	0.82	1.19	0.67	80.2	55.5
3	3.51	0.56	1.22	1.33	88.7	72.8
4	3.26	0.62	1.27	1.72	80.2	61.3

Table IV

Intracellular Distribution of Absorbed Phosphorus after Fermentation with Phosphate and Metal Ions

The yeast cells, inhibitable by phosphate, were fermented 3 hours with various additions, harvested, washed with cold water, fractionated, and the phosphorus was estimated. The phosphorus content of each of the fractions was compared with the corresponding quantities of cell material before and after fermentation, and the difference per 100 mg. of original dry cell material (ΔP) was calculated in each of the fractions.

Dssignations same as those in Table III.

Additions	ΔP (*mg.*)				ΔP (%to total phosphorus increase)	
	Pt	Po	Pm	Pmi	Po+Pm+Pmi	Pm+Pmi
Phosphate (0.05 *M*)	2.94	0.41	0.74	0.51	56.5	42.5
Phosphate (0.05 *M*) $+MgSO_4 \cdot 7H_2O$ (0.04 *g.*)	2.20	0.37	1.20	0.62	100.0	82.8
Phosphate (0.05 *M*) $+CaCl_2$ (0.02 *g.*)	2.97	0.63	1.31	0.38	63.3	56.9

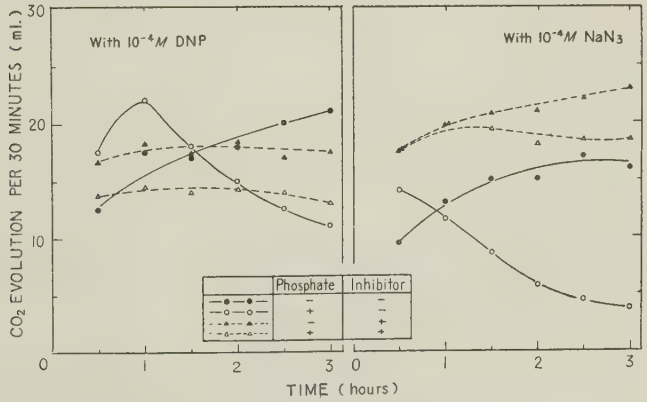


Fig. 6. Effect of 2,4-dinitrophenol and sodium azide on the fermentation rate with and without phosphate.

Temperature 30°, yeast 0.5 g., sucrose 2 g., citrate buffer 0.05 *M*, phosphate 0.05 *M*, inhibitor 10^{-4} *M*, water to 20 ml.

did not play any part in the fermentation rate. Moreover, in the case of cells not inhibited by phosphate, magnesium did not show any action on the fermentation rate.

It has been said that potassium ion plays an important role in the formation of metaphosphate (*10*), but it did not indicate any action in the present series of experiments.

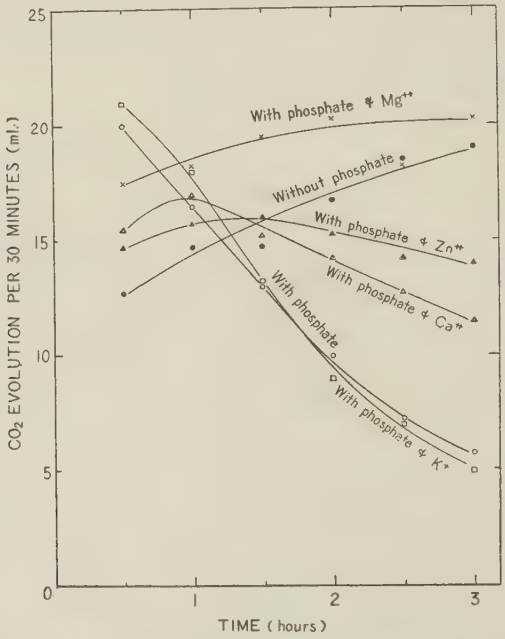


FIG. 7. Effect of metal ions on the fermentation rate with the presence of phosphate.

Temperature 30°, yeast 0.5 g., sucrose 2 g., citrate buffer 0.05 *M*, phosphate 0.05 *M*, metal ion, water to 20 ml. The quantity of metal ions are equivalent to 0.04 g. $MgSO_4 \cdot 7H_2O$; *i.e.* $CaCl_2$ 0.02 g., $ZnSO_4 \cdot 7H_2O$ 0.05 g., KCl 0.02 g.

Briefly stated, it was found that magnesium ion was effective not only on the inhibiting process but on the revival of inhibited cells, while calcium or zinc ion was effective only on the inhibiting process.

DISCUSSION

Nilsson and Alm (*1*) observed that phosphate suppressed the

fermentation rate of intact yeast cells. Their experimental conditions differed considerably from those of the present experiments, and there are also distinct differences in the observed phenomena between these two experiments. They used a considerably high concentrations of phosphate and observed a slight suppression of fermentation rate in all stages of fermentation, but they did not observe a sharp increase in the initial stage of the fermentation. At a relatively low concentration of phosphate in their experiments (about 0.08 *M*) there was scarcely any action on the fermentation.

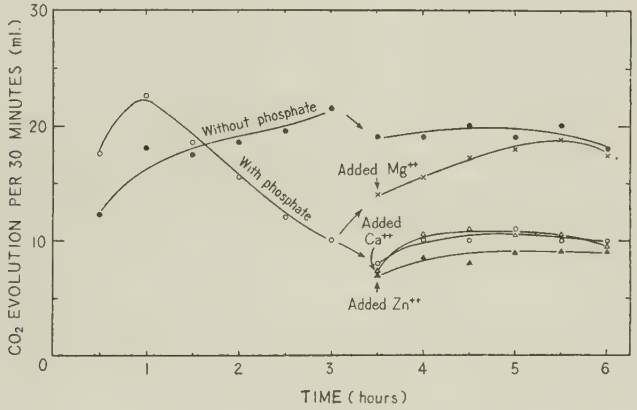


Fig. 8. Effect of metal ions on the rate of secondary fermentation. Temperature 30°, yeast 0.5 g., sucrose 2 g., citrate buffer 0.05 *M*, phosphate 0.05 *M*, water to 20 ml. After 3 hours of fermentation added some metal ions or water and again proceeded with fermentation. The quantity of metal irons are equivalent to 0.04 g. $MgSO_4 \cdot 7H_2O$, *i.e.*, $CaCl_2$ 0.02 g., $ZnSO_4 \cdot 7H_2O$ 0.05 g.

Atkin, Schultz, and Frey (*2*) observed a decrease in the fermentation rate when magnesium was omitted from their fermentation medium.[1] Further, Trevelyan and Harrison (*4*) found that phosphate caused a rapid decline in the fermentation rate at a later stage of fermentation, and magnesium and nitrogenous compound partially counteracted the phosphate action. However, the former workers did not make clear the rate decline due to the phosphate, and the latter merely described

1) Containing sucrose, phosphate, asparagine, magnesium, potassium, thiamine, pyridoxine, and nicotinic acid in citrate buffer.

the phenomenon.

The phenomenon in question was not observed commonly in all bakers' yeast cells, but it became clear from the above experiments (Figs. 4, and 6, Tables I, II, and III) that this fermentation inhibition was accompanied with intracellular accumulation of phosphate as metaphosphate when the phosphate-starved yeast cells were placed in a medium containing phosphate. In spite of the accumulation of much metaphosphate, fermentation inhibition was not observed in the presence of magnesium or calsium as is shown in Table IV, and Fig. 7, unlike in the case of dinitrophenol or sodium azide which inhibits the metaphosphate formation.

Metaphosphate has been considered as the phosphate-reservoir by some workers (*16*, *17*) and also recognized to bind with metal ions (*10*) or proteins (*11*). From the result of present experiments, it was assumed that metaphosphate inactivates the enzymes (a) by combining with them or (b) by combining with essential metal ions. In the former case, it may be explained that the metal ions deprive the metaphosphate of the imaginary enzyme-metaphosphate complex which is formed accompanied with the metaphosphate accumulation. It must be concluded in this case that only magnesium ion is effective and not zinc or calcium to dissociate enzyme from the enzyme-metaphosphate complex which has been formed at the time of metal ion addition. It seems more appropriate to explain by the latter case, namely that the metaphosphate catches the magnesium ion which is so indispensable to the fermentation process. Moreover, the inhibitory action of phosphate is counteracted completely by magnesium and partially by calcium or zinc during the process of metaphosphate formation. This indicates that the fermentation inhibition, which is probably caused by the deficiency of magnesium, will not occur if the metaphosphate in the process of formation combines with zinc or calcium ion instead of magnesium.

Nihei (*18*) reported interesting phenomena resembling somewhat the present observations and stated that photochemical reduction in *Chlorella ellipsoidia* was suppressed accompanying with the abnormal intracellular accumulation of metaphosphate in its ripening phase when it was synchronously cultured. When yeast cells were synchronously cultured,[2] the phenomenon in question could not be observed in any one of the phases of growth under normal cultural condition, in spite of variations in the phosphorus content of cells. However it may be a

2) Unpublished experiments.

phenomenon occurring in a certain physiological stage of yeast cells, namely, that of a phosphate-starved condition. The physiological meaning of this stage and of abnormal accumulation of metaphosphate is still uncertain.

SUMMARY

1. When some kinds of bakers' yeast cells were placed in phosphate-containing sugar solution, the rate of fermentation increased intensely at the initial stage and then rapidly decreased at a later stage, but the rate of respiration was not affected.

2. This fermentation inhibition was scarcely observed in low pH ranges.

3. The cells that accepted fermentation inhibition by phosphate did not recover the original fermentation rate on being merely washed of the external phosphate.

4. This phenomenon occurred not only in the phosphorus-poor cells but in the phosphorus-rich cells after phosphate starvation. The phosphorus-poor cells accumulated phosphate within the cells as metaphosphate during fermentation. 2,4-Dinitrophenol and sodium azide blocked this fermentation inhibition, which indicated the intimate relation between metaphosphate formation and fermentation inhibition.

5. Metal ions such as magnesium, calcium, and zinc counteracted this inhibition at the stage of metaphosphate accumulation, thereafter only magnesium released from this inhibition and calcium and zinc did not indicate any action.

6. It was inferred from the above observation that metaphosphate formed during fermentation had combined with magnesium, by which the rate of fermentation decreased.

The author would like to thank Mr. T. Shōda, the Director of Oriental Yeast Manufacturing Company, who gave him the opportunity to publish this report. The author is grateful for continued encouragement of Mr. K. Miyakawa, the chief of Research Department, and Dr. S. Nishimura, the former chief of this Department. The author wishes also to express his sincere gratitude to Mr. T. Matsuo for his technical assistance.

REFERENCES

(*1*) Nilsson, R., and Alm, F., *Biochem. Z.*, **286**, 254 (1936)
(*2*) Atkin, L., Schultz, A. S., and Frey, C. N., *Cereal Chem.*, **22**, 321 (1945)
(*3*) Atkin, L., and Gray, P. P., *Arch. Biochem.*, **15**, 303 (1947)

(4) Trevelyan, W. E., and Harrison, J. S., *Biochem. J.* (*London*), **57**, 556 (1954)
(5) Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925)
(6) Schmidt, G., and Thannhauser, S. G., *J. Biol. Chem.*, **161**, 83 (1945)
(7) Katchman, B. J., and Fetty, W. O., *J. Bacteriol.*, **69**, 607 (1955)
(8) Jeener, R., and Brachet, J., *Enzymologia*, **11**, 222 (1944)
(9) Schmidt, G., Hecht, L., and Thannhauser, S. J., *J. Biol. Chem.*, **166**, 775 (1946)
(10) Schmidt, G., Hecht, L., and Thannhauser, S. J., *J. Biol. Chem.*, **178**, 733 (1949)
(11) Juni, E., Kamen, M. D., Reiner, J. M., and Spiegelman, S., *Arch. Biochem.*, **18**, 387 (1948)
(12) Wiame, J. M., *J. Biol. Chem.*, **178**, 919 (1949)
(13) Yoshida, A., *Symposia on Enzyme Chem.*, **7**, 89 (1952)
(14) Yoshida, A., and Yamataka, A., *J. Biochem.* (*Tokyo*), **40**, 85 (1953)
(15) Spiegelman, S., Kamen, M. D., and Sussman, M., *Arch. Biochem.*, **18**, 409 (1948)
(16) Yoshida, A., and Yamataka, A., *Symposia on Enzyme Chem.*, **8**, 86 (1953)
(17) Hoffmann-Ostenhof, O., and Weigert, W., *Naturwissenschaften*, 39, 303 (1952)
(18) Nihei, T., *J. Biochem.* (*Tokyo*), **42**, 245 (1955)

The Journal of Biochemistry, Vol. 44, No. 9, 1957

ENZYMATIC PREPARATION OF OPTICALLY ACTIVE ESSENTIAL AMINO ACIDS

III. THE PREPARATION OF L-METHIONINE

By SHIGEKI YAMADA and SETSUJI SAKURAI

(From the Laboratory of Biochemistry, Faculty of Science, Osaka University, Osaka)

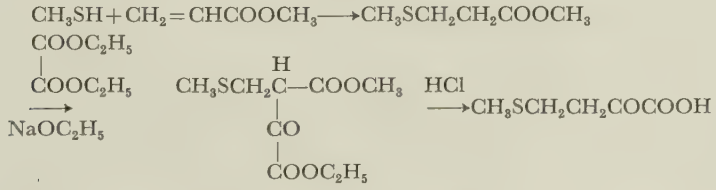
(Received for publication, April 19, 1957)

In the previous paper of tihs series (*1*), a method for the preparation of L-tryptophan by enzymatic transamination was described. In the present work, the same method was applied for the preparation of L-methionine.

It is well known that ketomethionine (keto analogue of methionine, *i.e.*, α-keto-γ-methylmecaptobutyric acid) has many interesting biological activities; Cahill (*2*) showed that methionine can be replaced by ketomethionine in the diet of animal and Handler (*3*) confirmed that ketomethionine has the same activity as L-methionine in the formation of creatine in rat liver slice.

Attempt to prepare ketomethionine has been made only enzymatically (*4*, *5*). Meister (*5*) obtained ketomethionine as sodium salt by oxidation of L-methionine with L-amino acid oxidase of rattle snake venom in an yield of 77 per cent.

For the purpose of development of the practical synthetic method of ketomethionine, we examined the applicability of the reaction as shown in the preparation of phenylpyruvic acid and indolepyruvic acid and confirmed that this method is suitable for large scale preparation of ketomethionine.



In the course of this experiment, we observed that 2,4-dinitrophenylhydrazone of ketomethionine is easily converted into an unknown sub-

stance when treated with commercial ethyl acetate and that the determination of ketomethionine by column chromatography (*6*) can be made only when ethyl acetate has been carefully purified.

The same phenomenon was observed in the determination of methionine when 2,4-dinitrophenyl derivative was treated with commercial ethyl acetate and we confirmed that 2,4-dinitrophenyl-methionine is easily oxidized to 2,4-dinitrophenylmethionine sulfoxide with impurity in commercial ethyl acetate.

In view of this fact, it appears that the same reaction undergoes in the determination of ketomethionine.

In this article, we describe the procedure for the preparation of ketomethionine and L-methionine and note on the determination of these substances.

EXPERIMENTAL AND RESULTS

Preparation of α-Keto-γ-methylmercaptobutyric Acid

Methyl β-Methylmercaptopropionate (*7*)—One hundred grams of methylmercaptan was dissolved in 400 g. of benzene and 2 g. of sodium methoxide was then added. To this mixture 198 g. of methyl acrylate was added with stirring in small portions so as to control the temperature at 5–8°. The solvent was removed and the residue was distilled at diminished pressure and the distillate was once redistilled. Methyl β-methylmercaptopropionate boiling at 79–80° under 15 mml Hg. weighed 237 g. (85 per cent).

Methyl α-Ethoxalyl-β-methylmercaptopropionate—One hundred and forty-six grams of diethyl oxalate was added to the alcoholic solution of sodium ethoxide (23 g. of sodium was dissolved in 280 g. of absolute ethanol) with vigorous stirring. After 30 minutes, 134 g. of methyl β-methylmercaptopropionate was added in a rapid stream. The mixture was heated in a water bath at 80° for 1 hour, then kept at this temperature for 3 hours at reduced pressure to expel ethanol. Ethanol was removed completely and the residue was taken up in 800 ml. of ice water. The solution was extracted twice with 300 ml. of ether (from the ether extract, 50 g. of methyl β-methylmercaptoprioponate was recovered) and the aqueous layer was acidified to pH 1.0 with hydrochloric acid and extracted five to eight times with an equal volume of ether. The combined ether solution was dried over anhydrous sodium sulfate and the ether was distilled off. The residue, mainly consisting of methyl α-ethoxalyl-β-methylmercaptopropionate weighed 170 g. (73 per cent).

Methyl α-Methoxalyl-β-methylmercaptopropionate—A solution of 12 g. of methyl α-ethoxalylmethylmercaptopropionate in 120 g. of methanol containing 2 per cent hydrochloric acid was refluxed for 2 hours and allowed to stand overnight. Removal of methanol gave 11 g. of methyl α-methoxalyl-β-methylmercaptopropionate.

2,4-Dinitrophenylhydrazone of this ester was prepared as reported previously (*1*). From 0.5 g. of this ester 0.8 g. of the crude hydrazone was obtained. After recrystallization from methanol containing a small amount of acetic acid, 0.4 g. of yellow crystals which melt at 117° was obtained.

Analysis	Calcd. for $C_{14}H_{16}N_4O_8S$	C, 42.04 ;	H, 4.19 ; N, 14.10
	Found	C, 42.02 ;	H, 4.18 ; N, 14.10

α-Keto-γ-methylmercaptobutyric Acid—A mixture of 11 g. of methyl α-methoxalyl-β-methylmercaptopropionate and 150 ml. of 5 perc ent hydrochloric acid was stirred at 70–75° until evolution of carbon dioxide has slowed considerably. This usually takes 7 hours under this condition. The reaction mixture was extracted several times with 250 ml. of ether and the ether solution was extradoted five times with 150 ml. of 5 per cent sodium bicarbonate solution and the aqueous layer was acidified to pH 1.0 with hydrochloric acid and then the solution was extracted several times with 250 ml. of ether. The combined ether solution was washed with a small amount of water and dried over anhydrous sodium sulfate and the ether was distilled off.

The residue, crude α-keto-γ-methylmercaptobutyric acid, weighed 40 g. (54 per cent). Chromatographic analysis (*6*) showed that this product wss 90 per cent pure.

The sodium salt was prepared as follows ; A solution of 2 g. of α-keto-γ-methylmercaptobutyric acid in 5 ml. of water was brought to pH 4.5 with 1 *N*-sodium hydroxide and concentrated in vacuo to about 10 ml., then 50 ml. of acetone was slowly added to it and the mixture was allowed to stand in refrigerator.

The crystalline sodium salt was filtered off, washed with cold acetone. After recrystallization with water-acetone, 1.2 g. (52 per cent) of sodium salt wss obtained.

2,4-Dinitrophenylhydrazone of α-keto-γ-methylmercaptobutyric acid was prepared as follows ; A mixture of 0.2 g. of the sodium salt of α-keto-γ-methylmercaptobutyric acid in 100 ml. of 0.2 per cent 2,4-dinitrophenylhydrazine solution wss allowed to stand overnight. The hydrazone was filtered, washed with cold water, once recrystallized from

methanol containing a small amount of acetic acid and dried in vacuo at 50°. The yellow crystals melting at 150–151° weighed 0.15 g.

Analysis Calcd. for $C_{11}H_{12}N_4O_6S$ C, 40.25 ; H, 3.69 ; N, 17.07
Found C, 40.16 ; H, 3.60 ; N, 16.92

Preliminary Test of Glutamic Acid-Methionine Transamination—To find out a suitable condition for the preparation of L-methionine by enzymatic transamination, several experiments on glutamic acid-methionine transamination were conducted. Table I shows the result when the pig heart extract was used as enzyme.

TABLE I

Yield of L-methionine by Transamination

Composition. / Exp. No.			I	II	III	IV
Sodium salt of ketomethionine		(μM)	250	250	250	250
Monosodium glutamate		(μM)	250	500	—	—
Enzyme	Weight of heart muscle (*g.*)		1	1	1	—
Reaction time			Amount of L-methionine and unreacted substrates			
20 hrs.	ketomethionine	(μM)	120	115	178	219
	L-methionine	(μM)	83	110	25	0
48 hrs.	ketomethionine	(μM)	48	20	168	225
	L-methionine	(μM)	105	125	35	0
	glutamic acid	(μM)	145	290	0	0

Total volume was adjusted to 5 ml. Incubation temperature 38° ; pH 7.6.

From this experiment it can be concluded that undesirable reaction occured on ketomethionine or L-methionine. Under the condition of No. II, only a small amount of ketomethionine was found in the reaction mixture after 48 hours, when L-methionine was found in an yield of about 50 per cent. We confirmed that the liver extract has the same activity as that of heart extract and that of the bacterial transaminase prepared from *B. subtillis* is lower.

Preparation of L-Methionine—Into a solution of 2.6 g. of sodium α-keto-γ-methylmercaptobutyrate and 5.7 g. of monosodium glutamate monohydrate in 100 ml. of water was added 180 ml. of the enzyme solu-

tion prepared as reported previously (*1*). The solution was adjusted to pH 7.5 with aqueous ammonia, filled up to 300 ml. with water and the mixture was incubated at 37° for 48 hours. The reaction mixture was heated at 80° for 10 minutes to coagulate the enzyme protein and filtered. The clear filtrate was treated with concentrated hydrochloric acid and evaporated *in vacuo* to dryness. To the residue was added 40 ml. of concentrated hydrochloric acid to dissolve L-methionine hydrochloride and to precipitate glutamic acid hydrochloride. From this hydrochloric acid solution, excess of hydrochloric acid was removed completely *in vacuo*. The residue was extracted with 30 ml. of ethanol and the solution was dried *in vacuo*. The residue was dissolved in 100 ml. of water and the solution was passed through the column packed with Amberlite IR-4B to remove hydrochloric acid and a small amount of glutamic acid. The effluent treated with charcoal was evaporated *in vacuo*. The crude product which weighed 1.05 g. was once recrystallized with 50 per cent methanol. The colourless leaflets melting at 273° weighed 0.6 g.

Analysis	Calcd. for $C_5H_{11}O_2NS$;	C, 40.25 ;	H, 7.43 ;	N, 9.39
	Found	C, 40.69 ;	H, 7.32 ;	N, 8.79

Specific rotation of this product, $[\alpha]_D^{20°}$ showed +23.8° (4.0 per cent in 3 *N* HCl, 2 d*m*, 20°). According to Greenstein (*8*), Specific rotation of L-methionine is 23.4° (2 per cent in 2 *N* HCl, 25°).

Note on the Determination of Ketomethionine and Methionine—For the purpose of the determination of ketomethionine we applied the method used for α-ketoglutaric acid (*6*). In this procedure we observed that the pure ketomethionine gave two bands on the silica gel column when commercial ethyl acetate, which can be used for the determination of α-ketoglutaric acid, was used for extraction of 2,4-dinitrophenylhydrazone of ketomethionine (Fig. 1, No. 4). In order to examine this phenomenon some experiments were conducted as shown in Fig. 1.

From this experiment it was confirmed that impurity in commercial ethyl acetate gives rise to undersiable reaction on 2,4-dinitrophenlyhydrazone of ketomethionine and the ratio of the optical density of band A to that of band B in the experiment No. 4 amounts to more than 6.

The same experiment was conducted in the determination of methionine as shown in Fig. 2.

From this experiment, it now seems clear that 2,4-dinitrophenylmethionine was easily oxidized to 2,4-dinitrophenyl-methionine sulfoxide with impurity, which may be supposed to be peroxide, in commercial ether or ethyl acetate and consequently it may be concluded that the

band A shown in Fig. 1 was 2,4-dinitrophenylhydrazone of ketomethionine sulfoxide.

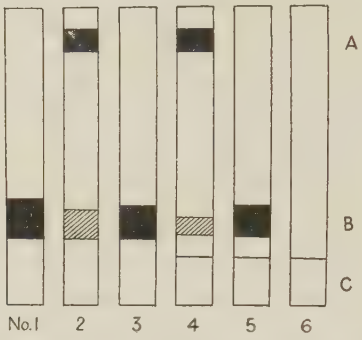


Fig. 1. Chromatogram of 2,4-dinitrophenylhydrozone of ketomethionine.

Column ; Silica gel was used (1.5 × 10 cm.).

Developing solvent ; A mixture of 3 volume of butanol and 97 volume of chloroform wss shaken with 0.5 per cent aqueous acetic acid and the lower layer was used.

Band A ; Unknown substance.

Band B ; 2,4-Dinitrophenylhydrazone of ketomethionine.

Band C ; 2,4-Dinitrophenylhydrazine.

No. 1 ; About 0.6 mg. of crystalline 2,4-dinitrophenylhydrazone of ketomethionine.

No. 2 ; About 0.6 mg. of crystalline 2,4-dinitrophenylhydrazone of ketomethionine was dissolved in 2 ml. of commercial ethyl acetate and dried *in vacuo*.

No. 3 ; The same conditions as in No. 2 except that purified ethyl acetate was used.

No. 4 ; 2 μM of sodium salt of ketomethionine was added to 3 ml. of 0.2 per cent 2,4-dinitrophenylhydrazine solution and allowed to stand for 20 minutes and the resultant hydrazone was extracted with 2 ml. of commercial ethyl acetate and then the solution was dried in vacume.

No. 5 ; The same conditions as in No. 4 except that purified ethyl acetate was used.

No. 6 ; 2 mol of commercial ethyl acetate was added to 3 ml. of 0.2 per cent 2, 4-dinitrophenylhydrazine solution and shaken for 2 minutes and the ethyl acetate solution was dried *in vacuo*.

In view of the above evidence, the solvents for extraction of 2,4-

dinitrophenylhydrazone of ketomethionine or 2,4-dinitrophenylmethionine should be carefully purified and kept always free from peroxide.

SUMMARY

1. α-Keto-γ-methylmercaptobutyric acid, keto analogue of methionine, was prepared by hydrolysis of methyl α-methoxalyl-γ-methylmercaptopropionate with dilute hydrochloric acid.

2. L-Methionine was obtained from ketomethionine by heart

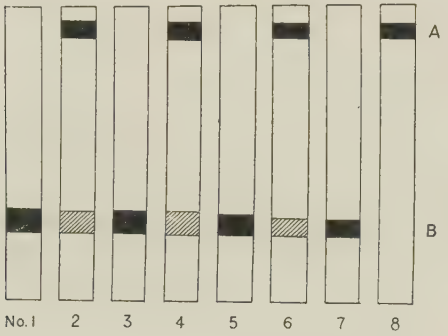


FIG. 2. Chromatogram of 2,4-dinitrophenyl-methionine.
Column and developing solvent ; The same as in Fig. 1.
Band A ; 2,4-Dinitrophenyl-methionine sulfoxide (*9*).
Band B ; 2,4-Dinitrophenyl-methionine.
No. 1 ; 3 μM of 2,4-dinitrophenyl-methionine.
No. 2 ; 3 μM of 2,4-dinitrophenyl-methionine was dissolved in 5 ml. of commercial ether and dried *in vacuo*.
No. 3 ; The same conditions as in No. 2 except that purified ether was used.
No. 4 ; The same conditions as in No. 2 except that commercial ethyl acetate was used instead of ether.
No. 5 ; The same conditions as in No. 4 except that purified ethyl acetate was used.
No. 6 ; 3 μM of methionine was reacted with 2,4-dinitrofluorobenzene and the reaction mixture was extracted with commercial ethyl acetate and then the solution was dried *in vacuo*.
N. 7 ; The same conditions as in No. 6 except that purified ethyl acetate was used.
No. 8 ; 2,4-Dinitrophenyl-methionine sulfoxide.

muscle transaminase in an yield of 46 per cent based on the ketomethionine used.

3. The effect of peroxide contained in commercial solvents on the determination of ketomethionine or methonine was described.

The author's best thanks are due to Prof. S. Akabori for his continued interest and advice.

REFERENCES

(*1*) Sakurai, S., *J. Biochem.*, **44**, 47 (1957)
(*2*) Cahill, W. M., and Rudolph, G. G., *J. Biol. Chem.*, **145**, 201 (1942)
(*3*) Handler, P., and Bernheim, M. L. C., *J. Biol. Chem.*, **150**, 335 (1943)
(*4*) Waelsh, H., and Borek, E., *J. Am. Chem. Soc.*, **61**, 2252 (1939)
(*5*) Meister, A., *J. Biol. Chem.*, **197**, 309 (1952)
(*6*) Sakurai, S., and Sugae, K., *J. Biochem.*, **42**, 367 (1955)
(*7*) Szabo J. L., and Stiller, E. T., *J. Am. Chem., Soc.*, **70**, 3667 (1948)
(*8*) Greenstein, J. P. *et al.*, *J. Biol. Chem.*, **194**, 455 (1952)
(*9*) Levy, A. L., and Chung, D., *J. Am. Chem. Soc.*, **77**, 2899 (1955)

The Journal of Biochemistry, Vol. 44, No. 9, 1957

QUANTITATIVE DETERMINATION OF LIPID-ETHANOLAMINE AND LIPID-SERINE AND THEIR DISTRIBUTION IN RAT AND PIG TISSUES

By SHOSHICHI NOJIMA and NOBUYUKI UTSUGI

(*From the Department of Biochemistry, National Institute of Health, Tokyo*)

(Received for publication, April 19, 1957)

Quantities of lipid-ethanolamine and -serine in organ extracts can be directly determined by Van Slyke's amino-N method (*1*) and also by his carbon dioxide determination method for α-amino acid nitrogen (*2*). But these methods are laborious and readily influenced by contaminants, such as urea, in lipid extracts. Recently these methods were criticized by Lea and Rhodes (*3*), who proposed a new spectrophotometric determination by ninhydrin (*4*). However, this method is not suitable for determining lipid-ethanolamine and -serine separately.

All the methods so far available for the fractional analyses of lipid-ethanolamine and -serine, involve the hydrolysis of lipid samples prior to the estimation of ethanolamine and serine in the hydrolysate.

These methods may be divided into two categories. The main procedure of the first one involves the reaction of the hydrolysate with alkaline periodate before and after ethanolamine is removed by permutit. Ammonia evolved is separated by steam distillation (*5*) or microdiffusion (*6*) and its quantity is determined. Quite recently McKibbin (*7*) described briefly a spectrophotometric determination method with 1,2-naphthoquinone-4-sulphonate after separation on a permutit column. The other method involves the formation of color derivatives of the hydrolysate with dinitrofluorobenzene, and the separation of the derivatives based on their solubility difference in organic solvents followed by the spectrophotometric determination (*8*). At the present time this latter method are widely used (*9*).

In the course of the study of liver phosphatides, it was strongly needed for us to assay lipid-ethanolamine and -serine by reliable and feasible method. The present paper describes an improved method, more sensitive and specific for estimation of lipid-ethanolamine and -serine. The authors also describe the results of the analyses of several

organ extracts of rat and pig by these methods and compare some of them with the previous data.

EXPERIMENTAL AND RESULTS

Reagent:

1. Dinitrofluorobenzene reagent (DNFB). 0.1 ml. of dinitrofluorobenzene (Wako Pure Chemical Industries, Ltd.) are dissolved in 2 ml. of 95 per cent ethanol. The reagent should be kept in a refrigerator.

2. 6 *N* hydrochloric acid, 2 *N* hydrochloric acid, 1 *N* hydrochloric acid. These solutions should be made by diluting 35 per cent hydrochloric acid (special grade) with water.

3. Standard ethanolamine solution. Ethanolamine is distilled to yield a fraction boiling at 168°. Working standards are prepared by dilution of the stock solution with water and directly titrated against standard acid using methylred as an indicator.

4. Standard serine solution. Working standards are prepared by dilution of the stock solution with water.

5. Xylene. Special grade should be used.

Total N, P and choline were determined by the methods of micro-Kjeldahl, Allen (*10*) and Glick (*11*), respectively.

Estimation of Ethanolamine and Serine in Aqueous Solution—One ml. of the standard solution, containing ethanolamine and serine both at 1 μM, and 1 ml. of water were taken in a 20 ml. glass-stoppered graduated test tube. 0.2 ml. of DNFB and 0.5 ml. of 5 per-cent aqueous $NaHCO_3$ solution were added, the tube was stoppered and warmed in a water bath at 80° for 1 hour with occasional shaking. After cooling, accurately 10 ml. of xylene was added, the tube was shaken vigorously for 5 minutes and then kept standing for further 10 minutes. The xylene layer contained the DNP-ethanolamine and the aqueous layer the DNP-serine. Accurately 8 ml. of the yellow xylene solution was transfered to a separatory funnel for DNP-ethanolamine assay.

DNP-serine was estimated as follows: After discarding the remaining (about 2 ml.) xylene layer about 8 ml. of xylene was added to the aqueous phase, the tube was shaken for a few minutes and left standing further for a few minutes. The upper layer was removed with Komagome pipette and discarded. This procedure was repeated again with the renewal of xylene. Thus the excess of DNP-ethanolamine was removed. The aqueous solution was acidified with 0.5 ml. of 1 *N* HCl and extracted with about 8 ml. of xylene three times in the same way as described above. The excess of DNFB (which may presumably be converted to dinitrophenol) was removed by these extractions. After the final removal of the solvent, accurately 2.5 ml. of the aqueous solution was transfered to a test tube and added 0.15 g. of $NaHCO_3$ powder together with 5 ml. of water. The optical density was determined at 400 mμ by Beckman spectrophotometer Model DU which should show 0.800.

DNP-ethanolamine was estimated as follows: 10 ml. of 6 *N* HCl and 30 ml. of petroleum ether were added to the above described separatory funnel containing 8 ml.

of DNP-ethanolamine xylene solution, and the funnel was shaken for a few minutes, then left standing for about 10 minutes. The optical density of the aqueous phase was determined at 420 mμ which should show 0.500.

A reagent blank run through the entire procedure should give a negligible optical density reading against distilled water.

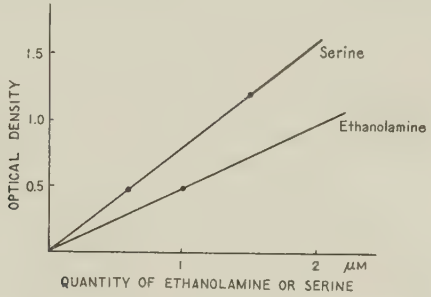


FIG. 1. Optical densities and amounts of ethanolamine and serine.

Fig. 1 shows the relationships between the amounts of ethanolamine and of serine and the optical densities of them indicating perfect linearity which extends beyond 2 μM of standards.

Scheme 1 shows schematically the outline of the above described procedure.

SCHEME 1

Outline of analytical Procedure

Hydrolysate of sample, 2 ml.
| DNFB
| $NaHCO_3$
Alkaline yellow solution
| Xylene

Xylene solution
| Petroleum ether HCl,

Xylene solution

Aqueous solution
DNP-ethanolamine
(420 mμ)

Aqueous solution
| Xylene

Xylene solution

Aqueous solution
| HCl
| Xylene

Xylene solution

Aqueous solution
| $NaHCO_3$
DNP-serine
(400 mμ)

Examination of Applicability to the Method of Various Solvents

While Axelrod employed methylisobutylketone and chloroform in extracting DNP-derivatives (*8*), xylene proved to be the most satisfactory solvent in this experiment. In order to select the best solvent for the assay, firstly, DNP-serine in an acidic aqueous medium was extracted with various organic solvents such as benzene, toluene, xylene, ethylene dichloride, chloroform, carbon tetrachloride, benzine, ligroin, carbon bisulfide and ethyl acetate, and losses of DNP-serine were examined. Among them only xylene and ligroin did not extract DNP-serine at all from the acidic aqueous phase when examined spectrophotometrically. Secondly, it was examined whether it is possible or not to remove with the solvent the excess of reagent, which should change to dinitrophenol after the reaction. Xylene extracted completely the excess of the reagent from the acidic medium after the reaction. Ligroin is somewhat inferior to xylene in this point. Eventually, it was found that xylene extracted only DNP-ethanolamine from the alkaline reaction mixture.

The reaction time and the reaction temperature as described above are the most suitable conditions in carrying out the assay.

Influences of the Contaminants in the Lipid-Hydrolysates upon the Assay

Urea 10 μM, sodium chloride 2 mM, choline chloride 10 μM, and inositol 10 μM gave no influence upon this method. Further, several common amino acids were tested because amino acids and peptides are known to be extracted along with lipids from tissues by organic solvents. Each of these amino acids was reacted with DNFB, fractionated in the same way as described in the estimation of ethanolamine and serine. All the amino acids examined, so far, produced DNP-derivatives which were not extracted by xylene in the alkaline media. Therefore, DNP-amino acids dissolved in acidified aqueous solution were treated with xylene and optical densities of the resulted aqueous solution were examined in alkaline media spectrophotometrically. The results are shown in Table I. The amino acids tested may be classified into three groups by the solubilities in xylene. The first group involves such amino acids as giving no influence on the assay. The second one shows about 10 per cent of the optical density of DNP-serine. DNP-derivatives of the last group furnish about the same optical densities as that of DNP-serine.

Application of this Method to the Analysis of Organ Extracts

Extraction and Purification—The lipids of rats were extracted and purified according to the procedures of McKibbin and Taylor (*12*). Five albino adult male rats

which had been maintained on stock ration were sacrificed by blows on the heads. The tissues were immediately removed, washed with water, and pooled. They were cut into conveniently sized pieces for grinding and homogenized with alcohol-ether (3:1) in a Waring blendor. After extraction with alcohol-ether and chloroform, both were combined and concentrated under reduced pressure in a stream of nitrogen. The residues were dissolved in chloroform and made up to 20 ml. The chloroform solution was purified with 0.25 *M* $MgCl_2$. After repeating of this purification nine times, the chloroform layers were transfered quantitatively to graduated test tubes and again

TABLE I

Optical Densities of Several DNP-Amino Acids in Alkaline Aqueous Media after Treatment with Xylene

Amino acids (1 μM.)	Optical density
Alanine	0.015
Valine	0.025
Leucine	0.0
Cysteine-HCl	0.024
Methionine	0.023
Phenylalanine	0.010
Lysine-2HCl	0.010
Tyrosine	0.0
Trypthophan	0.0
Glycine	0.065
Cystine	0.095
Proline	0.122
Serine	0.800
Histidine-HCl	0.385
Arginine	0.905
Oxyproline	1.195
Aspartic acid	0.835
Glutamic acid	0.690

made up to 20 ml. Aliquots from this solution were then used for analyses of total nitrogen, total phosphorous, choline, ethanolamine, and serine (Table II).

Phosphatides of pig liver were prepared as follows : Fresh pig liver weighed 1.3 kg. was freed of connective tissues as completely as possible and the tissue was minced with 1.4 litre of acetone in a Waring blendor. After leaving the extracts standing for a day and filtering off, the same procedure was repeated with the renewal of the solvent. The resulting residue was then extracted three times with 1.0 litre of petroleum ether. The combined petroleum ether extracts were concentrated to about 200 ml. under

reduced pressure in a stream of nitrogen, and left standing in an ice box. After centrifuging, the supernatant was poured into 500 ml. of acetone with stirring. Resolution of the precipitate in 100 ml. of ether and reprecipitation with 500 ml. of acetone, gave a yellow brown precipitate weighed 19 g. This solid emulsified in water was dialysed against distilled water for a couple of day and the dialyzed solution was lyophilized. Aliquots of the phosphatide were then analyzed in the same way as those of rat phosphatides (Table III).

Estimation of Lipid-Ethanolamine and -Serine in Organ Extracts—One ml. of each organ extract of rat was taken in a glass-stoppered test tube and chloroform was evaporated.

TABLE II

Several Analytical Data of Rat Tissue-Lipids

	N	P	N/P (molar ratio)	Choline	Ethanol-amine	Serine	Choline +Ethanolamine +Serine
Liver	132	134	0.99	52	21	10	83
Skeletal muscle	44	40	1.09	29	9	4	42
Heart	133	115	1.16	115	26	7	148
Lung	187	139	1.37	24	26	15	65
Kidney	191	170	1.12	68	40	14	122
Brain	558	449	1.24	141	120	49	310
Intestine	166	106	1.57	34	24	15	73

Values of N, P, choline, ethanolamine and serine are expressed in μM per g. dry lipid-free tissue residue.

TABLE III

Analytical Data of Pig Liver-Phosphatide

N (%)	P (%)	N/P (molar ratio)	Choline-N (%)	Ethanol-amine-N (%)	Serine-N (%)	Choline-N +Ethanolamine-N +Serine-N
1.85	3.38	1.21	0.99	0.33	0.17	1.49

In the case of pig phosphatides, about 2 or 3 mg. was weighed in the test tube. One ml. of 6 *N* NaOH was added and the tube was stoppered closely and placed in a boiling water bath for two hours. After cooling, the solution was made slightly acidic with 3 ml. of 2 *N* HCl, and the turbidity was clarified by means of a small amount of Celite and Norit. Two ml. of the solution was transfered to a 20 ml. glass-stoppered graduated test tube and ethanolamine and serine were determined in the same way as described above. The values obtained were multiplied by two.

Conditions of Hydrolysis—Artom (*5*) and Lovern (*13*) recommended the acidic hydrolysis of lipids because of the loss of ethanolamine and serine when proceeded in

the alkaline media. Lea (*14*) also used 6 *N* HCl because of the unreliability of the alkaline hydrolysis. On the other hand, Axelrod conducted hydrolysis of blood phosphatides with 2 *N* NaOH without any special description. On account of confused opinions about the hydrolysis conditions, the following experiments were carried out : in the first place, it was examined whether alkaline hydrolysis results in any loss of ethanolamine and serine or not. Heating of ethanolamine and serine with 2 *N* NaOH in a boiling water bath for two hours did not give any loss of both substances. Secondly the hydrolysis of the phosphatides of pig liver both in the acidic and alkaline conditions was carried out. (Fig. 2).

From these results the hydrolysis with 6 *N* NaOH gave the maximum values of both ethanolamine and serine. By hydrolysis with 6 *N* HCl for 21 hours a slightly lower value was obtained in the case of serine.

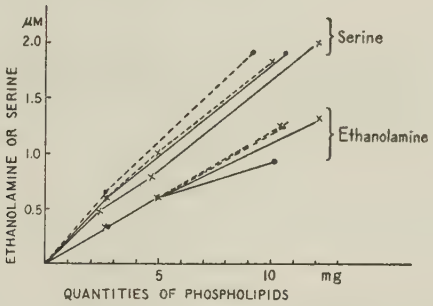


Fig. 2. Conditions of hydrolysis of phospholipids. —×— 6 *N* HCl, 3 hrs. --×-- 6 *N* HCl, 21 hrs. —●— 2 *N* NaOH, 2 hrs. --●-, 6 *N* NaOH, 2 hrs.

DISCUSSION

From the above described experiments, the present method offers the following several advantages to the Axelrod's method. (A) Instead of two solvents, chloroform and methylisobutylketone used by Axelrod, a single solvent, xylene was employed in this experiment. (B) Although the absolute specificity for ethanolamine and serine is still lacking because of similar behaviors like serine of several amino acids, this method is superior to previous ones. (C) Dinitrophenol is completely removed from the solution to be estimated. (D) Hydrolysis condition, 6 *N* NaOH for 2 hours, is selected because, for the time being, the maximum values should be chosen among the values obtained by various hydrolysis conditions, insofar as nature of nitrogen of lipids is not yet completely clear.

A number of data as to the amounts of total and choline-containing phospholipids in tissues of various animals, have been reported. On the other hand, only a few results of the previous literature can be compared with the present values for lipid-ethanolamine and -serine. Table IV shows the comparison between the previous and the present data on the ratios of the amounts of lipid-choline, -ethanolamine and -serine in liver of pig and rat (*15*, *16*). Taking into consideration of the differences in the degree of purification, the animal species and also the procedures employed, the values obtained here are fairly in good agreement with those reported by previous authors. The ratio of the amounts of lipid-choline, -ethanolamine and -serine in normal liver is 6–5:2.5–1.5:1.

TABLE IV

Comparison between the Previous and Present Data on the Ratios of the Amounts of Lipid-choline, -Ethanolamine and -Serine in Liver

Author	Animal	Fraction	Method	Choline	Ethanol-amine	Serine
Chargaff[1]	Pig	Whole tissue	Isotopic dilution method	7.1	4.1	1
Artom[2]	Rat	Whole tissue	$HJO_4$4-oxidation	5.0	2.4	1
				6.3	2.7	1
				8.1	4.3	1
McKibbin[3]	Rat	Nuclei	1,2-Naphtho-	4.9	2.2	1
		Mitochondria	quinone-4-sul-	4.5	1.6	1
		Microsome	phonate	5.8	1.7	1
		Supernatant		4.0	1.3	
Nojima	Rat[4]	Whole tissue	DNFB-method	5.1	2.1	1
	Pig[5]	Whole tissue		5.8	1.9	1

1) calculated from the percentages of total lipid N. (*15*)
2) calculated from the percentages of total phosphatides. (*17*)
3) calculated from the molar ratios of lipid component to P. (*16*)
4) calculated from the mole numbers.
5) calculated from the percentages of total lipid N.

SUMMARY

1. A new procedures with dinitrofluorobenzene using xylene as the fractionating solvent have been introduced in the method for the estimation of lipid-ethanolamine and -serine in animal tissues.

2. Lipid-choline, -ethanolamine and -serine have been determined in several tissues of rats and pig.

The authors wish to express their gratitude to Dr. D. Mizuno, Chief of the Department of Biochemistry, National Institute of Health, Tokyo, Prof. Dr. T. Ukita, Tokyo University, and Assistant Prof. Dr. Y. Yamakawa, Institute for Infectious Diseases, Tokyo, for their constant interests and encouragements throughout this work.

REFERENCES

(*1*) Van Slyke, D. D., *J. Biol. Chem.*, **9**, 185 (1911)
(*2*) Van Slyke, D. D., and Dillon, R. T., *C. R. Lab. Carsberg Serie Chim.*, **22**, 480 (1938)
(*3*) Lea, C. H., and Rhodes, D. N., *Biochim. et Biophys. Acta.*, **17**, 416 (1955)
(*4*) Lea, C. H., and Rhodes, D. N., *Biochem. J.*, **56**, 613 (1954)
(*5*) Artom, C., *J. Biol. Chem.*, **157**, 585 (1945)
(*6*) Burmaster, C. F., *J. Biol. Chem.*, **165**, 1 (1946)
(*7*) Spiro, M. J., and McKibbin, J. M., *J. Biol. Chem.*, **219**, 643 (1956)
(*8*) Axelrod, J., Reichenthal, J., and Brodie, B. B., *J. Biol. Chem.*, **204**, 903 (1953)
(*9*) Hayaishi, O., and Kornberg, A. *J. Biol. Chem.*, **206**, 647, (1954) ; Lea, C. H., Rhodes, D. N., and Stoll, R. D., *Biochem. J.*, **60**, 353 (1955)
(*10*) Allen, R. J. L., *Biochem. J.*, **34**, 858 (1940)
(*11*) Glick, D., *J. Biol. Chem.*, **156**, 643 (1944)
(*12*) McKibbin, J. M., and Taylor, W. E., *J. Biol. Chem.*, **178**, 17 (1949)
(*13*) Lovern, J. A. The Chemistry of Lipids of Biochemical Significance (Methuen & Co. London) (1955)
(*14*) Lea, C. H., and Rhodes, D. N., *Biochem. J.*, **56**, 617 (1954)
(*15*) Chargaff, E. Ziff, H., and Rittenberg, D., *J. Biol. Chem.*, **144**, 343 (1942)
(*16*) Spiro, M. J., and McKibbin, J. M., *J. Biol. Chem.*, **219**, 643 (1956)
(*17*) Artom, C., *J. Biol. Chem.*, **157**, 595 (1945)

The Journal of Biochemistry, Vol. 44, No. 9, 1957

DEGRADATION OF HEMIN IN THE SYSTEM OF DICYANHEMATIN-ASCORBIC ACID-H_2O_2

ON THE PURIFICATION OF THE REACTION PRODUCTS AND THEIR SPECTROSCOPICAL PROPERTIES

By KOOZOO KAZIRO, KEIZOO TSUSHIMA and FUJIKO UCHIMURA

(*From the Biochemical Laboratory, Nippon Medical School, Tokyo*)

(Received for publication, May 6, 1957)

A great variety of reaction systems leading to the oxidative decomposition of heme derivatives has so far been reported from several sites. Among these decomposition products which were recognized as spectroscopically detectable intermediates, only few have been inferred to be a single chemical entity, but not yet chemically disclosed though some presumable structures have been postulated by Lemberg *et al* (*1, 2, 3*). Also the chemical relationships between these intermediary compounds have not yet been established. For the further identification of these intermediates, each of these should be isolated in chemically pure form. Most of these compounds are, however, so unstable as to be isolated and identified.

The present authors have found in the reaction system of dicyanhematin, ascorbic acid and hydrogen peroxide, rather stable intermediary decomposition products (*4*), though the role of cyanide in this reaction process remains yet obscure. It will be reported in the present paper on the isolation procedure of these intermediary compounds and on their spectroscopic properties.

EXPERIMENTAL AND RESULTS

For the nomenclature of these intermediary compounds, provisional designations, 588-heme and 618-heme, will be used in the following description considering the optical properties of their cyanide compounds.

I. Isolation of 588-heme and its Spectroscopical Properties

A solution of dicyanhematin in a concentration of 5×10^{-5} *M* per litre, was prepared by dissolving recrystallized hemin in the mixture of

10 ml. of $N/10$ NaOH and 90 ml. of 0.6 M KCN solution. To 100 ml. of this solution, 10 ml. of 0.5 M ascorbic acid neutralized with NaOH and 5 ml. of 0.15 M H_2O_2 were added. After 80 minutes standing at room temperature the reaction were in completion producing a compound characterized by its absorption at 588 mμ in its ferrous state, which will be hereafter denoted as dicyanhemochrome of 588-heme.

(*a*) *Extraction of 588-heme*—After the reaction forming 588-heme compound was in completion, which could be confirmed spectroscopically, the reaction was interrupted by adding dithionite. Vigorously stirring, one third volume portion of glacial acetic acid was added to the reaction mixture and HCN was driven off by aeration. About one fourth volume portion of chloroform was then added and was shaken for 30 minutes to extract 588-heme compound into chloroform. The chloroform phase was washed several times with water to remove the remaining HCN in the solution. By repeating the washing and with the removal of HCN, the solubility of 588-heme in chloroform decreased gradually and finally it precipitated partially at the contact surface of chloroform and water. Precipitated 588-heme was taken out and the chloroform phase was separated from the water phase. Both precipitates and the chloroform solution were brought together and evaporated to dryness under reduced pressure. A portion of the isolated heme was dissolved in $N/10$ NaOH, KCN was added, reduced with dithionite and its absorbance was investigated. The absorption figure thus recorded coincided well with that of the initial reaction mixture at its final reaction stage.

Though the most part of the protoheme was converted into 588-heme, there remained apparently a small amount of the former in the reaction mixture unaffected. The remaining protoheme was separated from 588-heme by the following procedure.

(*b*) *Separation of Protoheme and 588-heme from their Mixture after Converting into their Porphyrins*—Protoheme and 588-heme in the reaction mixture were treated to remove their iron and converted into their porphyrins. These porphyrins could be separated from each other by making use of the difference in their HCl-number. Finally a pure 588-heme could be reconstructed from this porphyrin by introducing Fe into its molecule. The procedure will be described in the following.

A solid 588-heme obtained as above described containing a small amount of protoheme was dissolved in 10 per cent methanolic solution of oxalic acid. The iron could be removed from the heme molecule by introducing HCl gas into the solution after the addition of ferrosulfate

(*5*). Thus, porphyrin dimethylesters were formed in the methanolic HCl solution. The solution was strongly diluted with water and the esters were extracted in ether. The ether phase was washed with water several times and the porphyrin dimethylesters were then extracted in HCl-solution of various concentrations.

Since the HCl-number of protoporphyrin dimethylester was reported as 5.5 (*6*), the ether phase was shaken at first with 5.5 per cent HCl to remove the remaining protoporphyrin dimethylester. On this treatment, a certain portion of these porphyrinesters could be transfered into water phase which showed an absorption figure as illustrated

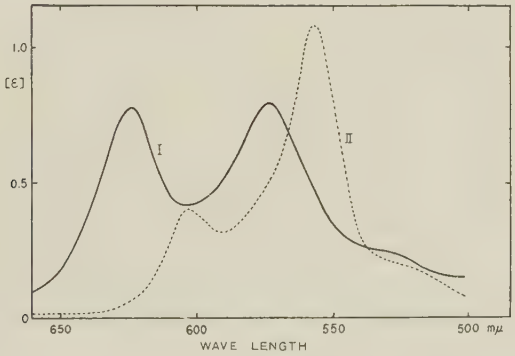


FIG. 1. Absorption figures of 588 mμ-porphyrin dimethylester and protoporphyrin dimethylester in 20 per cent HCl.

I. 588-porphyrin dimethylester. Absorption maxima ; 624 mμ, 573 mμ and (530 mμ)

II. Protoporphyrin dimethylester.

in Fig. 1, II with its maxima at 602 and 556 mμ coinciding well with that of protoporphyrin-HCl solution. By repeated extraction, the remaining protoporphyrin ester could be removed exhaustively from the ether phase.

On the next, the extraction was continued with more concentrated HCl-solution. With 10 per cent HCl solution only a small portion, but with 15 per cent HCl solution a most portion of porphyrin could be extracted. This fraction of porphyrin dimethylester was investigated spectroscopically in its 20 per cent HCl solution. The recorded absorption figure was shown in Fig. 1, I, the figure of 588-porphyrin dimethylester (as to be proved in the following description).

(*c*) *Spectroscopical Property of 588-porphyrin*—A small fraction of porphyrin which is extractable with 10 per cent HCl showed a similar absorbance. With 15 per cent HCl repeatedly extracted porphyrin fractions were collected. The color of this fraction was green.

The obtained 588-porphyrin ester was hydrolyzed with 5 per cent KOH solution and extracted in chloroform. The chloroform solution showed an absorption figure as given in Fig. 2. The maxima of the absorption were at 640 (I), 586 (II), 554 (III) and 514 mμ (IV). Pro-

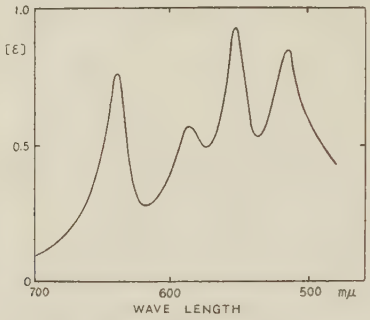


Fig. 2. Absorption figure of 588-porphyrin in chloroform. Absorption maxima ; 640, 586, 554 and 514 mμ.

toporphyrin has also four maxima in visible range. The absorption which is characteristic for 588-porphyrin compared with that of proto-porphyrin is that the absorptions I and III are relatively high and that the maxima are shifted rather toward the longer wave length side in general.

(*d*) *Reconstruction of 588-heme from 588-porphyrin*—Now to exclude the possibility of artificial alteration of the nucleus in porphyrin molecule as well as of its side chains during the course of the preparative procedure, a reconstruction of heme of this isolated porphyrin was conducted by introducing Fe into its molecule.

The porphyrin obtained from the fraction of 15 per cent HCl extraction wss dissolved in glacial acetic acid and heated with ferroacetate. The ferrous iron was easily incorporated into the porphyrin ring. After heating, the reaction solution was vigorously shaken with air in order to oxidize the heme-iron into its ferric state. Then it was extracted in ether. The remaining free porphyrin was removed from the ether solution extracting with 15 per cent HCl. The heme reconstructed

was then transfered into NaOH solution. To this hemin solution, KCN was added in an excess, reduced with dithionite and the absorbance was recorded. As illustrated in Fig. 3, I, an absorption maximum was found at 588-mμ, which is characteristic for the dicyanhemochrome of the new hemin. When pyridine was added to this alkaline hemin solution and reduced with dithionite, an absorption figure was obtained as shown in Fig. 3, II (dipyridine hemochrome of 588-heme).

In the absorption change taking place during the reaction process of 588-heme formation from dicyanprotoheme, clear cut isosbestic points were recognizable at 575 and 513 mμ. The ratio of the optical densities at these wave length were found to be 1.75 as reported in the

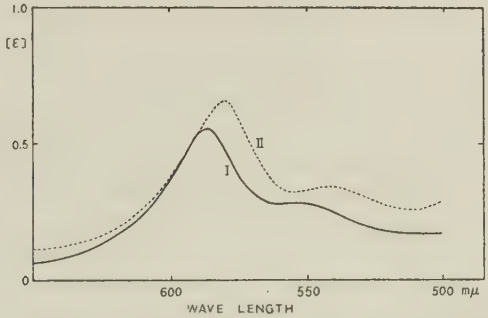


FIG. 3. Absorption figures of dicyan- and dipyridine hemochrome of 588-heme.

I. dicyanhemochrome. absorption maxima ; 588, 550 mμ.

II. dipyridine hemochrome. absorption maxima ; 580, 542 mμ.

previous paper (*4*). In the reconstructed 588-heme thus obtained, the ratio of the densities at 575 and 513 mμ was found similary to be 1.76. Thus it may be inferred that the absorption figure illustrated in Fig. 3, I is the absorption figure of dicyanhemochrome derived from the pure 588-heme which is produced from dicyanhematin in the present system and that the porphyrin of 588-heme could be obtained by no means artificially altered during the course of the preparative procedure. The absorption figures illustrated in Fig. 1, I and Fig. 2 are proved to be those of 588-porphyrin in HCl and in chloroform, respectively.

(*e*) *Molecular Extinctions*—The millimolar extinction coefficient ε_{mM}^{588} of the dicyanhemochrome of 588-heme thus obtainable in pure state can be given as follows:

$$\varepsilon_{mM}^{588}=\varepsilon_{588}/\varepsilon_{575}\times\varepsilon_{mM}^{575}$$

The ratio $\varepsilon_{588}/\varepsilon_{575}$ in dicyanhemochrome of 588-heme was found to be 1.5. The millimolar extinction coefficient of both dicyanprotoheme and dicyanhemochrome of 588-heme at 575 mμ (isosbestic point) was found to be $\varepsilon_{mM}^{575}=9.82$. By introducing these values into above equation, the millimolar extinction coefficient at 588 mμ for dicyanhemochrome of 588-heme could be calculated as $\varepsilon_{mM}^{588}=17.73$. By a similar treatment the millimolar extinction coefficient of pyridine hemochrome derived from 588-heme at 580 mμ could be obtained as $\varepsilon_{mM}^{580}=17.2$.

II. Isolation of 618-heme and its Spectroscopical Property

(*a*) *Isolation of 618-heme after Converting into its Porphyrin*—The second intermediate showing its absorption maximum at 618 mμ can be obtained under the same condition for the formation of 588-heme compound when allowed to stand in room temperature for a prolonged period, namely for 24 hours. This compound was isolated in the similar way as the former, transformed into its porphyrin by detaching its iron and the porphyrin was isolated by separating it from other porphyrins remaining in the mixture solution. The ether solution of the porphyrin thus obtained was extracted at first with 15 per cent HCl and a green porphyrin solution was obtained. The absorption figure of the extracted porphyrin dimethylester in HCl solution is shown in Fig. 4, I, which, compared with that of 588-porphyrin, shifted markedly toward longer wavelength side in general. The absorption figure of the new porphyrin is especially characterized by the fact that the second maximum at 588 mμ is far weaker than that of the first maximum at 643 mμ. On this point the new porphyrin is in clear distinction from 588-porphyrin. And the absorption figure of the chloroform solution also shifted toward longer wavelength side further.

(*b*) *Reconstruction of 618-heme from its Porphyrin*—The new porphyrin was dissolved in glacial acetic acid and iron was introduced into its molecule using ferroacetate. Then, dicyanhemochrome and dipyridine hemochrome was prepared from this new hemin. The absorbance of these compounds are shown in Fig. 6, I and II. Each of this absorption maximum was at 618 and 608 mμ, respectively. Thus it could be proved that the 618-porphyrin has remained unaltered even in this rather drastic treatment for the detachment of iron from the hemin. As the

fact, the absorption figure illustrated in Fig. 4, I and Fig. 5, I can be accepted as those of 618-heme in HCl and in chloroform solution, respectively.

(*c*) *Reaction of 618-heme Formation Starting from 588-heme*—Now, starting from the dicyanhemochrome of this pure 588-heme prepared as described above, the reaction was performed under the condition as mentioned in Fig. 7. As the reaction proceded, the absorption of the dicyanhemochrome of 588-heme decreased gradually giving rise of the new compound with its maximum at 618 mμ. As illustrated in the figure,

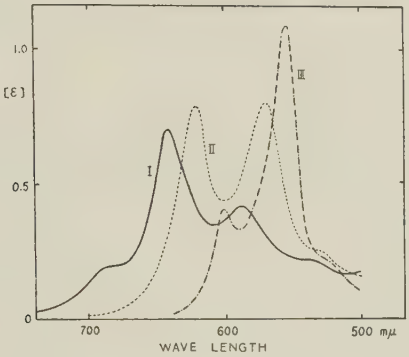


Fig. 4. Comparison in absorption spectra of 618-porphyrin dimethylester with 588- and protoporphyrin dimethylester in 20 per cent hydrochloric acid.

I. 618-porphyrin dimethylester. absorption maxima ; (690), 643, 588 mμ.

II. 588-porphyrin dimethylester.

III. protoporphyrin dimethylester

two clear cut isosbestic points at 604 and 532 mμ were established during the reaction was proceeding, indicating a reaction process of two reaction components.

It was thus evidenced again that the dicyanhemochrome of 588-heme is an intermediary product of the reaction from dicyanhematin into dicyanhemochrome of 618-heme.

(*d*) *Molecular Extinctions*—The millimolar extinction coefficient of the dicyanhemochrome of 618-heme at 618 mμ can be obtained from the following equation.

$$\varepsilon_{mM}^{618}=\varepsilon_{618}/\varepsilon_{604}\times\varepsilon_{mM}^{604}$$

Then, ε_{mM}^{618} was calculated as 10.02. By a similar treatment, the millimolar extinction coefficient of pyridine hemochrome derived from 618-heme at 608 mμ could be obtained as $\varepsilon_{mM}^{608}=9.48$.

DISCUSSION

(1) For an unstable intermediate produced in the reaction system of pyridinehemochrome-ascorbic acid-H_2O_2 leading to the formation of

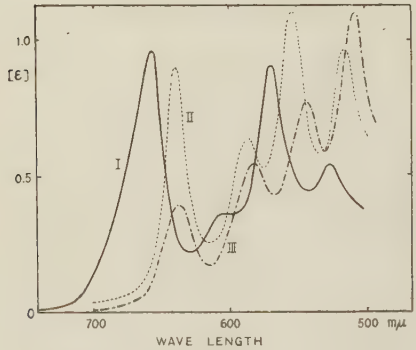


Fig. 5. Comparison in absorption figures of 618-porphyrin with 588- and protoporphyrin in chloroform.

I. 618-porphyrin

Absorption maxima ; 658, 605, 570, and 525 mμ.

II. 588-porphyrin

III. protoporphyrin

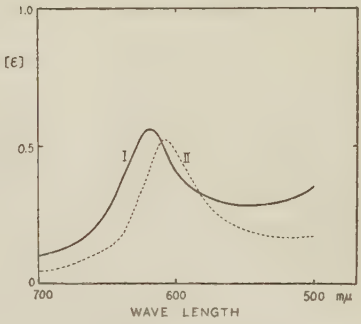


Fig. 5. Absorption figures of dicyan- and dipyridine-hemochrome of 618-heme.

I. dicyanhemochrome of 618-heme

II. dipyridine hemochrome of 618-heme

630 mμ-compound, a bile pigment precursor, Lemberg (*6*) suggested an oxyheme structure. Its porphyrin suggested as oxyporphyrin is green in its HCl solution. The absorption maxima of this porphyrin were reported as at 623.5 (I), 568.5 (II), its HCl-number as 0.25.

The new porphyrin, the 588-porphyrin which was obtained by the present authors shows two absorption maxima at 624 (I) and 573 mμ (II) in its HCl solution. In respect to the position of its absorption maxima, it is in close resemblance to the oxyporphyrin of Lemberg *et al.*, while its HCl-number was proved to be much higher than that of

oxyporphyrin.

From the comparison of the HCl-numbers, 588-porphyrin resembles rather to dioxy- or monoxymesoporphyrin of Fischer (7). The property of the new porphyrin coincided by no means with any of the known porphyrins hitherto described. The HCl-number of 618-porphyrin, a further oxidation product of 588-porphyrin, is greater than the latter. The absorption maxima of 618-porphyrin was found to be shifted rather toward longer wave length side in general.

It seems to be of special interest, that the ratio of the extinctions

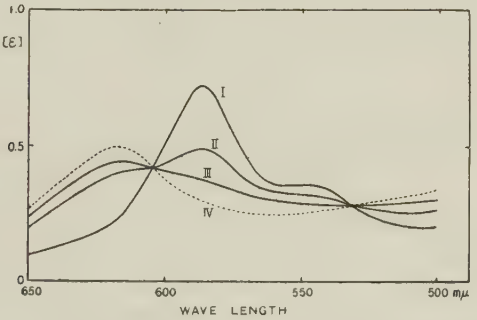


Fig. 7. Absorption change during the reaction process of 588-heme decomposition.

Concentration of 588-heme ; 4.9×10^{-5} *M*

Concentration of ascorbic acid

(neutralized with NaOH solution) 4.35×10^{-2} *M*

Concentration of hydrogen peroxide 6.25×10^{-3} *M*

I. 0 minute II. 20 minutes III. 150 minutes

IV. absorption figure of 618-heme obtained from Fig. 6, I.

of the first and the second maximum became reversed as the oxidation proceded as shown in Fig. 4.

The absorption maxima of some derivatives of these two intermediate hemes and the millimolar extinction coefficients of their dicyan- and dipyridine hemochromes are shown in Table I.

(2) A pyridine solution of 588-heme and 618-heme are both active as an oxidase in the same magnitude as pyridine-protohemochrome. Yet they do not transform in the presence of ascorbic acid and molecular oxygen into a biliverdin precursor as pyridine protochemochrome does. They are converted into some unknown product which does not give

TABLE I

Intermediate heme	Derivatives	Absorption maxima	ε_{mM}
588-heme	Dicyanhemochrome	588, 550	$\varepsilon_{mM}^{588}=14.73$
	Dipyridine-hemochrome	580, 542	$\varepsilon_{mM}^{580}=17.2$
	Porphyrin dimethylester (in 20% HCl)	624, 573, (530)	
	Porphyrin (in chloroform)	640, 586, 554, 514	
618-heme	Dicyanhemochrome	618	$\varepsilon_{mM}^{618}=10.02$
	Dipyridine-hemochrome	608	$\varepsilon_{mM}^{608}=9.48$
	Porphyrin dimethylester (in 20% HCl)	(690), 643, 588, (540)	
	Porphyrin (in chloroform)	658, 605, 570, 525	

rise of biliverdin. Further, the rate of the present reaction is much slower than that of the verdohemochrome formation. Thus, though these hemes can be decomposed peroxidatively, yet their reactivity with hydrogen peroxide seems much weaker than that of protoheme or 630 mμ-compound. Also it may be inferred that the reaction process of the peroxidative decomposition of these hemes are essentially different from that of verdohemochrome formation.

(3) When 588-porphyrin dimethylester was dissolved in small amount of warm methanol and allowed to stand in an ice box, it crystallizes in part in microscopical cubes. Its crystallization and purification are now in progress in the authors laboratory.

SUMMARY

1. Two intermediary products of heme decomposition in the system of dicyanhematin-ascorbic acid-hydrogen peroxide were isolated. They were designated tentatively as 588- and 618-heme from their spectroscopic properties.

2. The millimolar extinction coefficients of dicyanhemochromes and dipyridine hemochromes derived from 588- and 618-heme purely isolated were determined as $\varepsilon_{mM}^{588}=14.73$, $\varepsilon_{mM}^{580}=17.2$, $\varepsilon_{mM}^{618}=10.02$, and $\varepsilon_{mM}^{608}=9.48$.

3. By detachment of iron from these two hemes, 588- and 618-porphyrin were prepared and purified. Absorption maxima of 588-porphyrin dimethylester in 20 per cent HCl solution were at 624, 573 and (520) mμ. Those of 618-porphyrin dimethylester were at (690), 643, 588 and (540) mμ. In chloroform solution, 588-porphyrin showed its maxima at 640, 586, 554 and 514 mμ, while 618-porphyrin at 658, 605, 570 and 525 mμ.

The spectroscopical properties of these two products and their derivatives are summarized in Table I.

4. No biliverdin nor its precursor could be obtained from 588-heme nor 618-heme in the system of their pyridine-hemochrome-ascorbic acid-hydrogen peroxide.

5. In the present reaction, 588-heme has been proved to be the immediate precursor of 618-heme.

The present work was aided in part by the grant of Scientific Research Fund of the Ministry of Education for which the authors wish to thank.

REFERENCES

(*1*) Lemberg, R., Cortis-Jones, B., and Norrie, M., *Biochem. J.*, **32**, 149 (1939); **32**, 171 (1938)

(*2*) Lemberg, R., Legge, J. W., Hematin Compounds and Bile Pigments, p. 453, Interscience Publ., N. Y. (1949)

(*3*) Lemberg, R., Falkes, E. C., and Purdom, P., *Proc. Roy. Soc., London* **B 138**, 386 (1951)

(*4*) Tsushima, K., Kikuchi, G., Makita, M., Uchimura, F., and Kaziro, K., *J. Biochem.*, **44**, 525 (1957)

(*5*) Grinstein, M., and Watson, C. J., *J. Biol. Chem.*, **167**, 515 (1947)

(*6*) Lemberg, R., Legge, J. W., Hematin Compounds and Bile Pigments, p. 69, Interscience Publ., N. Y. (1949)

(*7*) Fischer, H. and Orth, H., Die Chemie des Pyrrols, p. 269–274, Erste Hälfte, Akadem. Verlagsgesellschaft, Leipzig (1937)

The Journal of Biochemistry, Vol. 44, No. 9, 1957

DENATURATION AND INACTIVATION OF ENZYME PROTEINS

V. DENATURATION AND INACTIVATION OF YEAST ALCOHOL AND MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES*

By ICHIRO SEKUZU, BUNJI HAGIHARA, FUMIO HATTORI, TAKESHI SHIBATA,** MITSUHIRO NOZAKI and KAZUO OKUNUKI

(*From the Department of Biology, Faculty of Science, University of Osaka, Osaka*)

(Received for publication, May 6, 1957)

As previously reported in this series (*1–4*), the " bacterial proteinase " method for the determination of the " ratio of denaturation " of partially denatured globular proteins has been demonstrated to be very useful in detecting any modifications in the intramolecular structure of enzyme proteins such as bacterial and fungous amylases and catalase. Using this method, the inner-structural change of two DPN-linked dehydrogenases, yeast ADH and muscle GDH, produced by the addition of PCMB or MIA was studied in the present paper, because the necessity of SH groups for the catalytic action of these dehydrogenases has been recognized by many investigators. At the same time, the process of denaturation of these enzymes was also examined in the presence of urea which was known as one of typical denaturing reagent of proteins. Thus, the difference in the mode of action of these reagents was distinguished with the respect for relationship between denaturation and

---

* This work was reported at the 7th Meeting of Symposia on Enzyme Chemistry, Japan, in Nagoya, July, 1954.

** Present Address : Faculty of Fisheries, University of Hokkaido, Hakodate, Hokkaido.

*** The following abbreviations are used in the present and following two papers : ADH, alcohol dehydrogenase : GDH, glyceraldehyde-3-phosphate dehydrogenase ; LDH, lactic dehydrogenase ; DPN, DPN^+, and DPNH ; Diphosphopyridine nucleotide, oxidized form, and reduced form, respectively ; ATP, adenosine triphosphate ; AMP, adenosine monophosphate ; TCA, trichloroacetic acid ; MIA ,monoiodoacetate ; PCMB, p-chloromercuribenzoate ; SH, sulfhydryl ; " RD ", "ratio of denaturation " ; " RI ", " ratio of inactivation ".

inactivation of these dehydrogenases.

MATERIALS

Crystalline ADH was prepared from baker's yeast according to the method of Racker (*5*) and recrystallized once. Crystalline GDH was prepared from rabbit skeletal muscle by the method of Cori, Slein, and Cori (*6*) and recrystallized four times. The crystallization of GDH was carried out in the presences of 0.01 *M* of cysteine and versene by the slightly modified method of Velick *et al.* (*8*). DL-Glyceraldehyde used as the substrate of GDH was kindly supplied by Dr. Y. Ogura of the University of Tokyo. DPN^+ of about 90 per cent purity was prepared from baker's yeast by the method of Okunuki *et al.* (*7*). For the determination of denaturation, BPN′ (crystalline proteinase of *B. subtilis* N′) was used (*4*). Other materials were the same as those described in the preceding papers (*1–4*).

METHODS

Preparation of the Enzyme Solution—The crystals of ADH and GDH were dissolved in 0.01 *M* phosphate buffer (pH 7.4) and in 0.01 *M* sodium arsenate-HCl buffer (pH 7.6), respectively, and dialysed overnight against each buffer solution in a refrigerator. After removing a slight amount of insoluble material by centrifugation, each dialyzate was diluted to approximately 1 per cent with each buffer.

Denaturation and Inactivation Treatments—The above ADH solution was incubated with an equal volume of urea or SH inhibitors, whereas the GDH solution was mixed with one half volume of the above reagents and "RD" and "RI" were measured using the aliquots pipetted out at suitable intervals. The other test conditions will be described in details at each section.

Determination of "RD" of ADH—To 0.5 ml. of the above reaction mixture of ADH was added 2 ml. of 0.1 per cent solution of BPN′ containing 0.2 *M* phosphate buffer (pH 7.5) and the mixture was incubated at 30° for 10 minutes. Then 1.2 *M* TCA was added and further incubated for 1 hour. The resulting precipitate suspension was filtered off through a toughened filter paper and 2 ml. of the filtrate was mixed with 2 ml. of 1.2 *M* Na_2CO_3 and 0.5 ml. of 1/5 Folin reagent. The optical density of the mixture was measured at 660 mμ after the incubation at 40° for 20 minutes. The sample completely denatured by heating at 80° for 5 minutes was also measured in the same way. The "RD" of ADH protein in a test solution was calculated according to the equation 2 in the 2nd report (*2*).

Determination of "RD" of GDH—The determination procedure of the "RD" of GDH was the same as in the case of ADH except that the digestion by the proteinase was carried out at 20° for 15 minutes and the color of Folin reaction was developed with 1 ml. of the filtrate, 5 ml. of 0.45 *M* Na_2CO_3, and 0.5 ml. of Folin reagent. A completely denatured sample was prepared by heating a GDH solution at 60° for 10 minutes in the presence of 3 *M* urea and then treated with the proteinase as above.

Assay of ADH Activity—The above reaction mixture was diluted 400 times with 0.01 *M* phosphate buffer (pH 7.0) containing 0.1 per cent gelatine. The enzymatic activity was determined approximately at 20° in a 1.0 cm. quartz cuvette in Shimadzu spectrophotometer type QB-50. The assay system contained, 10^{-2} *M* sodium pyrophosphate-HCl buffer (pH 8.6), 3×10^{-2} *M* ethyl alcohol, 4.3×10^{-4} *M* DPN^+, and 0.5 ml. of the diluted enzyme solution, and the total volume was 3.5 ml. The reaction was initiated by adding the enzyme solution with rapid mixing and the increments of optical density at 340 mμ were recorded at 20-second intervals. The difference between the 20- and 60-second readings was defined as the enzyme activity. " RI " was calculated from the equation 1 (*2*).

Assay of GDH Activity—The activity was also evaluated spectrophotometrically with a test system contained, 3×10^{-2} *M* sodium pyrophosphate-HCl buffer (pH 8.8), 10^{-2} *M* sodium arsenate, 3.2×10^{-2} *M* DL-glyceraldehyde, 4×10^{-4} *M* DPN^+, and 0.5 ml. of the reaction mixture of GDH. The total volume of the test system was 3.5 ml. and the temperature approximately at 20°. The reaction was initiated by adding the enzyme solution and the increase in density at 340 mμ was recorded at 30-second intervals. The difference between the 30- and 90-second readings was defined as the enzyme activity.

Special Procedure for Proteinase Digestion—When the reaction medium of GDH denatured with urea was diluted with the proteinase solution for assay of " RD ", the denatured GDH coagulated and the hydrolysis with the proteinase became difficult, while only a little coagulation occurred in the case of the denaturation by SH inhibitors. The error by the coagulation was completely eliminated by the following procedure, *i.e.*, to the test solution was added 1 ml. of the proteinase solution and the mixture was incubated for 10 minutes in order to digest most of denatured GDH while preventing it from coagulation, and then another 1 ml. of the proteinase was added for the complete digestion after further 10 minutes' incubation.

For " RI " in urea denaturation, the activity was measured in the test system containing 2 *M* urea which protected the solution from coagulation. The measurements were carried out within a short time such as the enzyme did not suffer from the urea. Since the coagulation was not completely prevented even by the above procedure, the error caused by turbidity was eliminated by using the same enzyme solution as a blank.

RESULTS

Effect of Proteinase—When native protein of ADH or GDH was incubated with proteinase, the decreases in enzymatic activities and that in protein concentrations precipitated with TCA were not observed even after incubation of 1 hour. On the contrary, the proteins completely denatured were rapidly digested by proteinase to the state almost soluble in TCA. Therefore, the proteolytic method for the determination of the " RD "s of GDH and ADH was useful with a small error caused by the above incomplete digestion of denatured enzymes.

Effect of Urea—As illustrated in Fig. 1, the time courses of denaturation and inactivation of ADH with 2 *M* urea at 30° proceeded according to unimolecular reaction and both ratios were almost coincided with

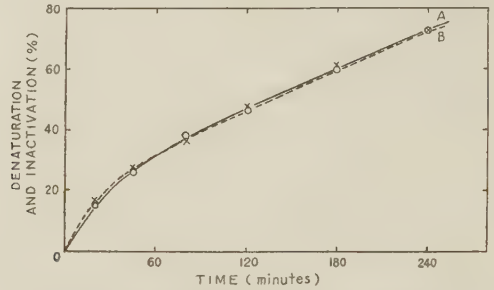


FIG. 1. Course of inactivation and denaturation of crystalline alcohol dehydrogenase in treatment with urea. The experiment was carried out in 2 *M* urea at 30°.

A. —○— Ratio of inactivation B. --×-- Ratio of denaturation

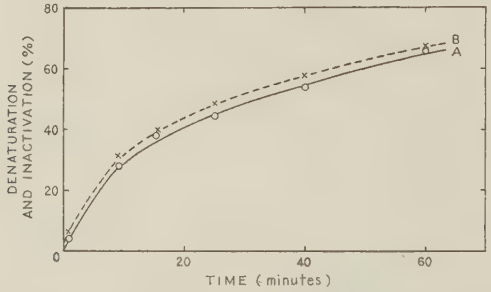


FIG. 2. Course of inactivation and denaturation of crystalline glyceraldehyde-3-phosphate dehydrogenase from rabbit skeletal muscle in the presence of urea.

The test system contained 2.5 *M* urea, 6.7×10^{-3} *M* sodium arsenate-HCl buffer, pH 7.6, and about 0.5 per cent enzyme. Temperature, 19.5°.

The method is described in the text and a special procedure was used in this experiment.

A. —○— Ratio of inactivation B. --×-- Ratio of denaturation

one another at every stage in the duration. In the case of the urea denaturation of GDH tested in 2.5 *M* urea at 19.5°, the almost similar result was also observed (Fig. 2). These results show that the inactiva-

tions of ADH and GDH by urea were caused only to the denaturation of the enzyme protein as in the cases of amylases and catalase (*1–4*).

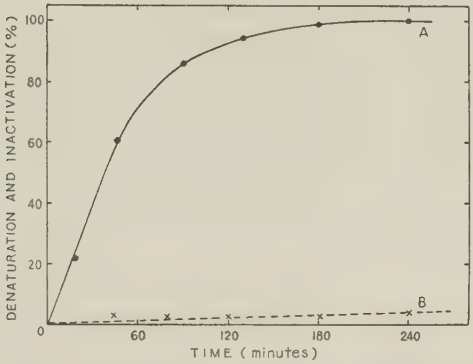


FIG. 3. Course of inactivation and denaturation of crystalline alcohol dehydrogenase in the presence of monoiodoacetate.

The experiment was carried out in 1.3×10^{-4} *M* MIA at pH 7.6 and at 30°. The method is described in text.

A. —●— Ratio of inactivation B. --×-- Ratio of denaturation

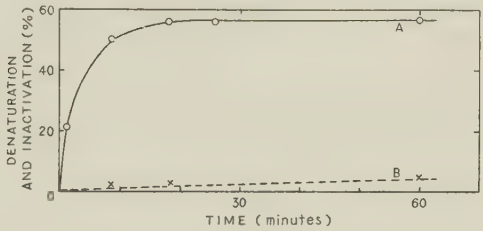


FIG. 4. Course of inactivation and denaturation of crystalline glyceraldehyde-3-phosphate dehydrogenase in the treatment with monoiodoacetate.

The experiment was carried out in 5×10^{-5} *M* MIA at pH 7.6 and the temperature was 21°. The detailed method is described in the text.

A. —○— Ratio of inactivation B. --×-- Ratio of denaturation

Effect of MIA—When ADH and GDH were incubated with MIA, they were remarkably inactivated, increasing with the time of incubation. Contrariwise, denaturation has hardly been observed. Such results are illustrated in Figs. 3 and 4.

Effect of PCMB—Fig. 5 illustrates the time courses of inactivation and denaturation of ADH in the presence of 3.3×10^{-4} *M* PCMB. Immediately after addition of PCMB, inactivation of ADH occurred to about 80 per cent, and then gradually increased. Denaturation also occurred, but the values of " RD " was always less than that of " RI ".

As illustrated in Fig. 6, on addition of 8.4×10^{-4} *M*, 6.25×10^{-5} *M*, and 5×10^{-5} *M* PCMB into GDH solution, the activity decreased to 30, 45, and 70 per cent, respectively, within 15 minutes, then partially recovered for about 20 minutes, and finally reached to the constant values which depended upon the concentration of PCMB. In the case

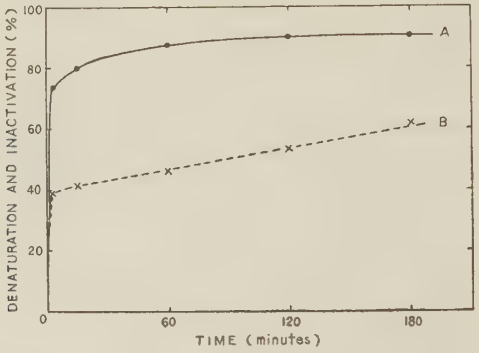


FIG. 5. Course of inactivation and denaturation of crystalline alcohol dehydrogenase by *p*-chloromercuribenzoate.
The experiment was carried out in 3.3×10^{-4} *M* PCMB at 30°.
A. —●— Ratio of inactivation
B. --×-- Ratio of denaturation

of lower concentration of PCMB, it was observed that the above automatic reversal of inactivation occurred to the greater extent. The denaturation by PCMB also occurred accompanying the inactivation, but in general, " RD " was fairly smaller than " RI ". In some cases where PCMB was added in low concentrations (Curve C and c in Fig. 6), " RD " and " RI " almost coincided with one another after that the " RI " became constant.

DISCUSSION

From the results illustrated in Figs. 1 and 2, it was confirmed that

urea acts on the protein moiety of ADH and GDH and causes inactivation of the enzymes by altering the secondary intramolecular structure, *i.e.*, denaturation, of the proteins as reproted previously about amylases and catalase (*1–4*). However, the effect of two SH inhibitors, MIA and PCMB, on both enzymes differed from that of urea. By the addition of MIA, strong inactivation was observed without denaturation, whereas denaturation which was fairly less than the inactivation proceeded in the case of PCMB treatment (Fig. 5). Since the denaturation was not observed in the presence of MIA, this inhibitor seems to have no effect on the protein moiety of both enzymes similar to the inactiva-

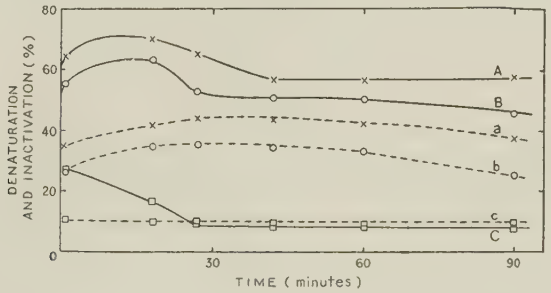


FIG. 6. Course of inactivation and denaturation of glyceraldehyde-3-phosphate dehydrogenase in the presence of various concentrations of *p*-chloromercuribenzoate.

The concentrations of PCMB used in this experiment were 8.4×10^{-4} *M* (Curves A and a), 6.25×10^{-5} *M* (Curves B and b), and 5×10^{-5} *M* (Curves C and c). The experiment was carried out in 6.7×10^{-3} *M* sodium arsenate-HCl buffer, pH 7.6, at 21°. Solid lines (indicated by capital letters) show the ratio of inactivation and dotted lines (indicated by small letters) the ratio of denaturation.

tion of catalase with specific inhibitors such as cyanide, fluoride, azide, and hydroxylamine (*4*). On the contrary, denaturation with PCMB shows that some modifications were caused in the protein moiety, but the modification is thought to be smaller than that with urea. Therefore, the inactivation may be caused mainly to the blocking of SH groups by PCMB and the combination of PCMB with enzyme might cause the alteration of the protein moiety. Thus, the difference in the mode of action between MIA and PCMB on the constellation of enzyme proteins was distinctly demonstrated in the case of ADH and GDH, and it was also shown through this investigation that the check of the effect of in-

habitors against intramolecular structure of enzyme proteins is necessary in determining the mode of action of inhibitors on enzymes as discussed in the 4th report of this series (*4*).

SUMMARY

Inactivations and denaturations of yeast ADH and muscle GDH were investigated in the presence of urea and two SH inhibitors, MIA and PCMB. The " ratio of denaturation " (" RD ") and the " ratio of inactivation " (" RI ") almost agreed with one another at any stage in the duration of urea treatment. In the case of PCMB treatment, however, " RI " was larger than " RD ", while, with MIA, denaturation was hardly observed in spite of the strong inactivation.

REFERENCES

(*1*) Okunuki, K., Hagihara, B., Matsubara, H., and Nakayama, T., *J. Biochem.* (*Japan*), **43**, 453 (1956)
(*2*) Hagihara, B., Nakayama, T., Matsubara, H., and Okunuki, K., *ibid.*, **43**, 469 (1956)
(*3*) Hagihara, B., Nakayama, T., Matsubara, H., and Okunuki, K., *ibid.*, **43**, 483 (1956)
(*4*) Hagihara, B., Shibata, T., Sekuzu, I., Hattori, F., Nakayama, T., Nozaki, M., Matsubara, H., and Okunuki, K., *ibid.*, **43**, 495 (1956)
(*5*) Racker, E., *J. Biol. Chem.*, **184**, 313 (1950)
(*6*) Cori, G. T., Slein, M. W. and Cori, C. F., *J. Biol. Chem.*, **173**, 605 (1948)
(*7*) Okunuki, K., Hagihara, B., Sekuzu, I., Nozaki, M., and Nakai, M., *J. Biochem.* (*Japan*), **42**, 389 (1955)
(*8*) Velick, S. F., Hayes, J. E., and Harting, J., *J. Biol. Chem.*, **203**, 527 (1953)

The Journal of Biochemistry, Vol. 44, No. 9, 1957

DENATURATION AND INACTIVATION OF ENZYME PROTEINS

VI. EFFECT OF DIPHOSPHOPYRIDINE NUCLEOTIDE ON DENATURATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*

By MITSUHIRO NOZAKI, ICHIRO SEKUZU, BUNJI HAGIHARA, TAKASHI YONETANI, MASASHI NAKAI AND KAZUO OKUNUKI

(*From the Department of Biology, Facutly of Science, University of Osaka, Osaka*)

(Received for publication, May 6, 1957)

In the previous paper (*5*), difference in the mode of action of three inhibitors, MIA**, PCMB and urea, on ADH or GDH was described, and it was noted that partial denaturation of dehydrogenases occurred by the addition of PCMB. Since the effect of PCMB is related to DPN-specific SH groups of GDH according to the work of Velick (*7*), it will be thought that the denaturation by PCMB must also have a relation with DPN binding of GDH.

In the present paper, action of DPN to protect GDH from the attacks of MIA, PCMB, and urea was studied using the " bacterial proteinase " method and presented the evidence for the stabilization of intramolecular structure of the enzyme protein which was caused by the presence of DPN.

MATERIALS AND METHODS

The DPN-free GDH was prepared by treating a crystalline GDH solution with activated charcoal (Norit A) according to the method of Taylor *et al.* (*6*). The other materials and methods were the same as described in the previous reports (*1–5*).

RESULTS

Urea Denaturation—For the purpose of clarifying the protective effect

* This work was reported at the 7th Meeting of Symposia on Enzyme Chemistry, Japan, in Nagoya, July, 1954.

** The abbreviations used in the present paper are the same as those described in the 5th report (*5*).

of DPN+ against urea denaturation of GDH, DPN-free GDH was used. The experiment was carried out in 3 *M* urea at 19.5° and the concentration of DPN+ added to each solution wss respectively 0, 100, and 500 μg. per ml. The same experiment but omitting the addition of 100 μg. per ml. of DPN+ was also carried out by using the same amount of charcoal-untreated GDH containing 90.2 μg. of DPN+ per ml. as the bound DPN+ with GDH. In assaying the enzymatic activity, the error caused from the difference of concentration of DPN+ was eliminated by adjusting the total amount of DPN− in each assaying system to final 4.3×10^{-4} *M*.

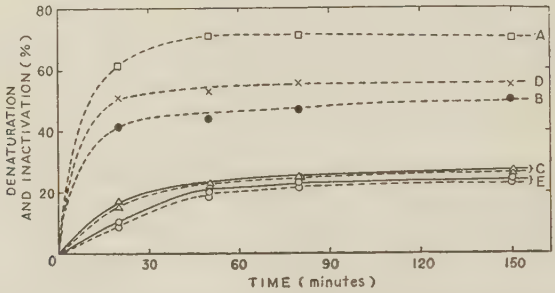


FIG. 1. Effect of DPN on urea denaturation of glyceraldehyde-3-phosphate dehydrogenase.

The test system contained 8.2 mg. protein per ml. of GDH treated with or without active charcoal, 3 *M* urea and 6.7×10^{-3} *M* sodium arsenate-HCl buffer, pH 7.6, and the temperature was 19.5°. The charcoal-untreated GDH contained 90.2 μg. per ml. of DPN+. This experiment used the special procedure (*5*). In Curves A, B, and D, the ratio of inactivation was not measured. The solid lines in the figure indicate the ratio of inactivation and the dotted lines the ratio of denaturation. A, charcoal-treated GDH+urea ; B, A+DPN+ (100 μg./ml.); C, A+DPN+ (500 μg./ml.); D, charcoal-untreated GDH+urea ; E, D+DPN+ (500 μg./ml.).

As illustrated in Fig. 1, the larger the amount of DPN+ present in a test solution, the smaller "RD" and "RI" were observed regardless of the charcoal treatment.

Inactivation by MIA—The time courses of inactivation and denaturation of GDH with 5×10^{-6} *M* MIA were measured under a similar experimental condition as mentioned in the foregoing section. As illustrated in Fig. 2, inactivation of the charcoal-untreated GDH proceeded without connection to the presence and absence of DPN+. In the inactivation of charcoal

treated GDH, a slight difference was observed between the presence and absence of DPN^+, but it was too small to consider as the protective action of DPN^+. As can be seen in Fig. 2, no denaturation was observed in any of the cases.

Denaturation and Inactivation by PCMB—The inactivation and denaturation by 8×10^{-5} *M* PCMB were observed in the absence and presence of 0.5 and 2.0 mg. per ml. of DPN^+. As illustrated in Fig. 3, almost the same course of inactivation was observed in all cases after 20 minutes, whereas the denaturation apparently decreased in the presence of DPN^+. This protective effect of DPN^+ against denaturation by PCMB was detected not

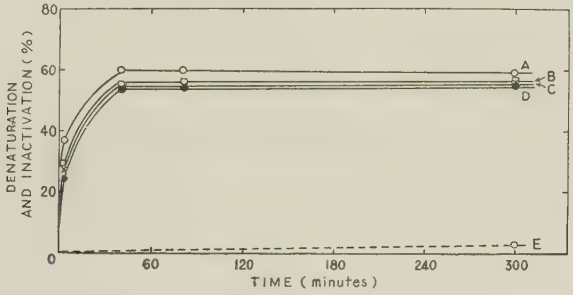


FIG. 2. Effect of DPN on glyceraldehyde-3-phosphate dehydrogenase in solution of monoiodoacetate.

Condition of treatment was 5×10^{-6} *M* MIA, at pH 7.6 and at 21°. The GDH was the same as in Fig. 1. Curve A, charcoal-treated GDH+MIA ; Curve B, A+DPN^+ (500 μg./ml.) ; Curve C, charcoal-untreated GDH+MIA ; Curve D, C+DPN^+ (500 μg./ml.). Curves A, B, C, and D show the ratio of inactivation and Curve E shows the ratio of denaturation of all the cases.

only by the proteolytic method, but also by the retarded appearance of coagulation caused by denaturation of the proteins which was shown by an arrow in the figure.

DISCUSSION

It was shown from the above results that inactivation by PCMB or MIA was not reduced by the presence of DPN^+, while a marked decrease of denaturation by urea or PCMB was observed by the coexistence of DPN^+.

The "RD" of the enzyme proteins so far experimented in the

previous work (*1–5*) almost agreeded with the "RI" in the duration of urea treatment, so it seems fairly obvious that urea denaturation of a protein is caused by the rupture of hydrogen bonds in the protein molecule. Therefore, the protective effect of DPN^+ against urea denaturation of GDH may suggest that the binding of GDH molecule with DPN^+ makes their intramolecular structure more rigid, in such a way that alteration of the protein moiety by rupture with urea becomes difficult. The stabilization of carboxylase or D-amino acid oxidase (*8*) by their coenzyme was already reported, so it may be a fairly common phenomenon that the stabilization of apoenzymes is caused by the binding with their coenzymes. Muscle GDH is known as a typical example

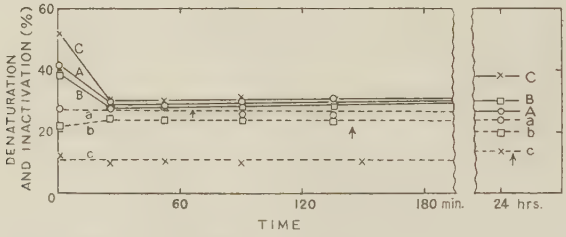


FIG. 3. Effect of DPN on denaturation and inactivation of glyceraldehyde-3-phosphate dehydrogenase in solution of *p*-chloromercuribenzoate.

Condition of treatment was 8×10^{-5} *M* PCMB, at pH 7.6 and at 21°. The solid lines (indicated by capital letters) show the ratio of inactivation and the dotted lines (indicated by small letters) the ratio of denaturation. Charcoal-treated GDH was not used. Curves A and a, no addition of DPN^+; Curves B and b, $+DPN^+$ (0.5 mg./ml.); Curves C and c, $+DPN^+$ (2 mg./ml.).

of coenzyme-binding protein, because the enzyme is not able to crystallize without DPN^+ and becomes very labile when the bound DPN is released from the enzyme (*6*).

In the case of PCMB denaturation, a similar protective effect of DPN^+ was also observed. As was found by Racker and Krimsky (*9*) and by Velick and Hayes (*10*), PCMB removes DPN from the protein molecule and this change is reversible in the presence of cysteine or reduced glutathione. However, denaturation was also found together with inactivation and the protective effect of DPN^+ was only observed against the denaturation process, so it is supposed that PCMB produces some changes in the configuration of protein moiety by combination with

SH groups of the protein as mentioned in a preceding report (*5*).

According to the result of Segel and Boyer (*11*), 1 mole of this protein contains 15 or 16 moles of SH groups, and 3 groups are active against DPN and 5 groups bind with MIA, among which 2 groups are specifically protected by glyceraldehyde-3-phosphate. From these facts, it is suggested that the differences in the mechanisms between denaturation and inactivation by PCMB is as follows: The automatic reversal of inactivation, which is observed in the first stage of the process by PCMB (*5*), shows that PCMB has stronger affinity to DPN-specific SH groups than to other SH groups. Since the protein moiety becomes more rigid by the presence of excess of DPN^+ and the combination of DPN-nonspecific SH groups with PCMB is inhibited, DPN^+ protects the protein from denaturation but from inactivation. By considering the above facts together with the effect of MIA on GDH, it may throw a light upon the mechanism of enzyme action.

Summarizing the above results, it appears that DPN is necessary not only for the appearance of enzyme action, but also for the stabilization of intramolecular structure of GDH molecule.

SUMMARY

Effect of DPN^+ against GDH in the presence of urea, MIA or PCMB was studied using the proteolytic method presented by us. Denaturation of GDH caused by urea or PCMB was markedly decreased by the presence of DPN^+, whereas inactivation caused by PCMB or MIA was not influenced by it. Based on the above results, the protective effect of intramolecular change of GDH caused by the addition of DPN^+ was discussed in connection with the difference of the mode of action of SH inhibitors.

REFERENCES

(*1*) Okunuki, K., Hagihara, B., Matsubara, H., and Nakayama, T., *J. Biochem. (Japan)*, **43**, 453 (1956)

(*2*) Hagihara, B., Nakayama, T., Matsubara, H., and Okunuki, K., *ibid.*, **43**, 469 (1956)

(*3*) Hagihara, B., Nakayama, T., Matsubara, H., and Okunuki, K., *ibid.*, **43**, 483 (1956)

(*4*) Hagihara, B., Shibata, T., Sekuzu, I., Hattori, F., Nakayama, T., Nozaki, M., Matsubara, H., and Okunuki, K., *ibid.*, **43**, 495 (1956)

(*5*) Sekuzu, I., Hagihara, B., Hattori, F., Shibata, T., Nozaki, M., and Okunuki,

K., *ibid.*, **44**, 587, (1957)
(*6*) Taylor, J. F., Velick, S. F., Cori, C. F., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, **173**, 619 (1948)
(*7*) Velick, S. F., *J. Biol. Chem.*, **203**, 563 (1953)
(*8*) Kubo, H., *Symposia on Enz. Chem. (Japan)*, **5**, 1 (1950)
(*9*) Racker, E., and Krimsky, I., *J. Biol. Chem.*, **198**, 731 (1952)
(*10*) Velick, S. F., and Hayes, J. E., *ibid.*, **203**, 545 (1953)
(*11*) Segel, H. L., and Boyer, P. D., *ibid.*, **204**, 265 (1954)

The Journal of Biochemistry, Vol. 44, No. 9, 1957

DENATURATION AND INACTIVATION OF ENZYME PROTEINS

VII. EFFECT OF COENZYME AND SUBSTRATES ON DENATURATION OF DEHYDROGENASES*

By ICHIRO SEKUZU, JINPEI YAMASHITA, MITSUHIRO NOZAKI, BUNJI HAGIHARA, TAKASHI YONETANI and KAZUO OKUNUKI

(*From the Department of Biology, Faculty of Science, University of Osaka, Osaka*)

(Received for publication, May 6, 1957)

During the course of investigation on the relation between denaturation and inactivation of two DPN-linked dehydrogenases, yeast ADH** and muscle GDH, it wss found that the dehydrogenases were protected from denaturation by DPN^+ (*5*, *6*). The protective effect of the substrates and coenzymes on the enzymatic activities of D-amino acid oxidase and glutamic dehydrogenase was reported by Kubo *et al.* (*14*) and Burton (*15*), but it was not clear whether they protect the active groups or structure of the enzyme proteins as a whole. Using the bacterial proteinase method for the determination of the denaturation of enzyme proteins (*1–6*), it was made clear by us that DPN stabilizes the secondary intramolecular structure of dehydrogenases.

In order to obtain a more detailed information of these protective effects against denaturation not only by urea but also by heat, following investigations were carried out: (a) effect of DPN on another dehydrogenase, LDH, (b) effect of substrate, (c) effect of coexistence of DPN and substrates, and (d) effect of some compounds which have a chemical structure related to DPN. The present communication deals with the effect on heart LDH and yeast ADH mentioned above and presented an evidence for the formation of a ternary complex of DPN and substrates with the dehydrogenases. The significance of the complex formation with respect to the mechanism of stabilization of the dehydrogenase will be described.

---

* This work was reported at the 8th Meeting of Symposia on Enzyme Chemistry, Japan, in Osaka, July, 1955.

** The abbreviations used in the present paper are the same as described in the 5th report (*5*).

MATERILAS

Crystalline LDH was prepared from beef heart by the slightly modified method of Straub (*7*) and recrystallized four times. Yeast ADH was crystallized by the method of Racker (*8*). These crystalline enzymes were lyophilized after dialysis against 0.01 *M* Na_2HPO_4-NaH_2PO_4 buffer, pH 7.6, for 4 hours. A commercial preparation of crystalline bacterial proteinase, " Nagarse ", was used for the determination of denaturation, which was the same enzyme as BPN′ used in the previous reports (*5*, *6*).

DPN^+ used in the experiments was a preparation of approximately 90 per cent purity, which was prepared by the method of Okunuki *et al.* (*9*) and followed by a further purification on a column of Duolite A-40 using 0.1 *M* ammonium-acetate buffer, pH 5.9. DPNH was prepared from DPN^+ by enzymatic reduction with yeast ADH and ethyl alcohol, and precipitated with excess of alcohol and dried in vacuum. Ba-ATP of about 75 per cent purity was prepared by the modified method of Dubois *et al.* (*10*). Sodium pyruvate was synthesized according to Clift and Cook (*11*) and recrystallized twice from aqueous alcohol. Na-AMP, L-lactic acid, and sodium pyrophosphate used were commercial preparations, and adenine was a kind gift of Mr. Y. Sugino, the Takeda Research Laboratory, Osaka. Na_2HPO_4-NaH_2PO_4 buffer was used in all studies unless otherwise specified. Other materials were described in the preceding papers (*1*–*6*).

METHODS

Preparation of Enzyme Solution—Lyophilized preparations of LDH, ADH, and proteinase were dissolved in 0.05 *M* buffer, pH 7.6, and immediately used for the experiments. The amount of LDH was calculated from the extinction coefficient at 280 mμ of 1.49 for 1 mg. per ml. in a 1-cm. cuvette according to Neilands (*17*).

Assay of Enzyme Activity—The activity of LDH was determined spectrophotometrically with a Shimadzu spectrophotometer type QB-50, in terms of the rate of decrease of optical density at 340 mμ, the absorption peak for DPNH. All the experiments were carried out with final concentrations of 3.8×10^{-4} *M* DPNH, 1.8×10^{-2} *M* sodium pyruvate, and 3.0×10^{-2} *M* buffer, pH 7.6. One-half milliliter of LDH solution, which was diluted 51 times the original test solution with 0.05 *M* buffer, pH 7.6, was placed in a cuvette and the reaction was initiated by the addition of DPNH solution while mixing vigorously. The total volume was 3.5 ml. and temperature approximately 25°. The reading of initial optical density at 340 mμ of the test cuvette was adjusted to one-half of that added with DPNH by the addition of sodium pyruvate into the control cuvette. The velocity constant (k_1) of the decrease in density at 340 mμ was calculated at 2-minute intervals according to the equation of unimolecular reaction, and the mean value during the first 12 minutes was defined as the enzyme activity. From Fig. 1 which illustrates the relation between the velocity constant and the amount of enzyme, the present method was confirmed to be very suitable for the determination of activity of LDH.

The assay of ADH activity was made by the same procedure described in the previous report (*5*).

" RI " was calculated from the equation 1 in the previous report (*2*).

Determination of " RD " of LDH—The main procedures were similar to those described in the previous paper (*5*), but they were slightly modified as follows : One milliliter of the test LDH solution was added to 2 ml. of 0.2 per cent solution of the bacterial proteinase and incubated for 20 minutes at 30°. After the incubation, 2.0 ml. of 1.0 *M* TCA was added and further incubated for 30 minutes. The concentration of digested product being soluble in 0.4 *M* TCA was measured by Folin's color reaction as before (*5*). In order to determine the value of a completely denatured sample, the same aliquot of the test solution was denatured by heating at 100° for 20 minutes and treated with proteinase solution as above.

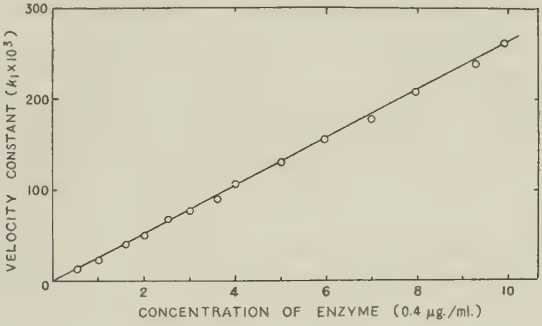


FIG. 1. Relationship of concentration of lactic dehydrogenase, in terms of final protein concentration, to the rate of reaction. The rate of reaction is expressed by the velocity constant (k_1) which was calculated from the decrease in optical density at 340 mμ.

The method was described in text.

" RD " of LDH protein in a test solution was calculated according to the equation 2 (*2*).

Determination of the Number of SH Groups in LDH—The number of LDH-SH groups reactive during denaturation was amperometrically measured according to the method of Kolthoff *et al.* (*12*) and Ingram (*13*).

Experimental Conditions of Denaturation—These will be described in each section of the Results.

RESULTS

I. Effect of the Substrates and DPN on Urea Denaturation of LDH

When LDH solution was treated with the proteinase at neutral

pH, a small amount of digestion was detectable, but a decrease in enzymatic activity was hardly observed. This slight digestion will be caused from a small amount of denatured protein contained in the crystals of LDH but the determination of " RD " was not affected by this impurity since the value of this impurity was eliminated as a value of control. On the contrary, the denatured LDH was rapidly digested to a state soluble in TCA by the bacterial proteinase.

(*A*) *Effect of DPN*$^+$ *and Pyruvate*—Fig. 2 Curve A illustrates the time courses of denaturation and inactivation of LDH in 3 *M* urea at 25°. The process of denaturation and inactivation proceeded in parallel and " RD " and " RI " at every stage were practically coincidental with one another. From the above result, it was concluded that the inactivation of LDH by urea was caused only from the denaturation of the enzyme protein. By the addition of DPN$^+$, " RD " and " RI " decreased considerably, while the parallelism of both values still existed (Fig. 2, Curve B). The effect of the addition of pyruvate was small (Fig. 2, Curve C). On the other hand, as illustrated in Fig. 2, Curve D, a marked protective effect which was larger than the sum of each protective effect given by DPN$^+$ and pyruvate was observed by the coupling of both substrates.

In Table I, the velocity of urea denaturations under similar conditions is indicated, in which the values of denaturation velocity are expressed as the reaction rate constant (k_1) at the first 15 minutes calculated as unimolecular reaction for the sake of convenience. The marked protective effect by this coupling was clearly demonstrated from the data.

This coupled protective effect against urea denaturation of LDH was also demonstrated by the amperometric measurement of the number of reactive SH groups (Fig. 3).

Since the agreement of " RD " and " RI " at every stage of urea denaturation was observed in all cases, only " RI " was measured in the following experiments.

(*B*) *Effect of Concentration of DPN*$^+$—As illustrated in Fig. 4, 10^{-3} *M* DPN$^+$ has a strong protective effect against urea denaturation, but 5×10^{-5} *M* DPN$^+$ had no effect. A small amount of the effect appeared by the addition of 10^{-4} *M* DPN$^+$.

(*C*) *Effect of DPNH*—A stronger protective effect was given by DPNH than by DPN$^+$, 10^{-4} *M* DPNH showed a strong protective effect and even 10^{-5} *M* DPNH had a marked effect (Fig. 5).

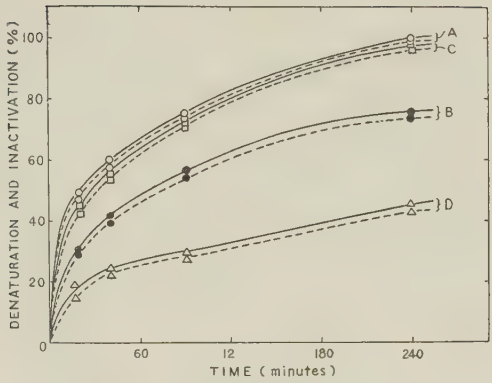


FIG. 2. Time course of inactivation and denaturation of crystalline lactic dehydrogenase from heart muscle in the presence of urea.

The experiments were carried out in the presence of 3 *M* urea and 1.5×10^{-5} *M* LDH at pH 7.0 and at 25°. Other conditions and methods were described in text. The solid lines show the ratio of inactivation and the dotted limes the ratio of denaturation measured by the proteinase method. Curve A, 3 *M* urea ; Curve B, A+1.5×10^{-3} *M* DPN^+ ; Curve C, A+1.5×10^{-3} *M* pyruvate ; Curve D, A+1.5×10^{-3} *M* DPN^++1.5×10^{-3} *M* pyruvate.

TABLE I

Velocity of Urea Denaturation of Lactic Dehydrogenase

The velocity of urea denaturation is expressed as the reaction rate constant (k_1) at the first 15 minutes calculated as unimolecular reaction.

Experimental No.	1	2
Experimental Condition	Urea 3 *M*, pH 7.6, 30° LDH 1.5×10^{-5} *M*	Urea 3.2 *M*, pH 7.6, 25° LDH 2×10^{-6} *M*
	k_1 at 15 min.	k_1 at 15 min.
No addition	0.015	0.022
+ 1.5×10^{-3} *M* DPN^+	0.008	0.016
+ 1.5×10^{-3} *M* pyruvate	0.014	0.021
+ 1.5×10^{-3} *M* DPN^+ + 1.5×10^{-3} *M* pyruvate	0.005	0.002

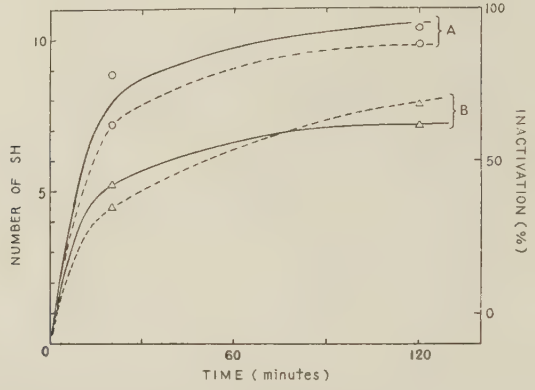


FIG. 3. Relationship between increment of reactive SH groups and inactivation of LDH in the case of urea denaturation.

The test condition was 3.2 *M* urea, 3×10^{-5} *M* LDH, at pH 7.6 and at 25°. The method was described in text. The solid lines show the ratio of inactivation and the dotted lines the number of reactive SH per molecule of the enzyme. Curve A, 3.2 *M* urea ; Curve B, A+3×10^{-2} *M* DPN^++3×10^{-3} *M* pyruvate.

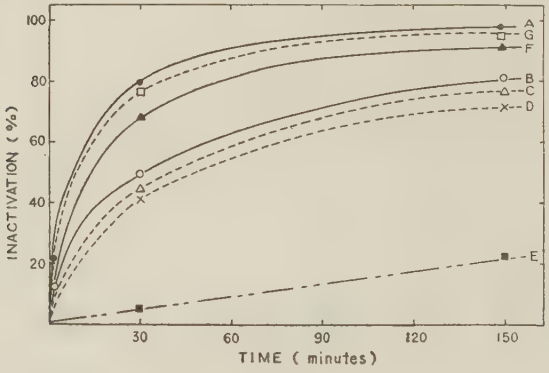


FIG. 4. Protective effect of DPN^+ and pyruvate in various concentrations on inactivation of lactic dehydrogenase in the presence of urea.

The test conditions were 3.2 *M* urea, 10^{-6} *M* LDH, at pH 7.6 and at 25°. Curve A, control (without DPN^+ and pyruvate) ; Curve B,+10^{-3} *M* DPN^+ ; Curve C, +10^{-3} *M* DPN^++10^{-4} *M* pyruvate ; Curve D,+10^{-3} *M* DPN^++10^{-3} *M* pyruvate ; Curve E,+10^{-3} *M* DPN^++10^{-2} *M* pyruvate ; Curve F, +10^{-4} *M* DPN^+ ; Curve G, +5×10^{-5} *M* DPN^+.

(*D*) *Effect of Lactate*—10^{-2} *M* Lactate showed no protective effect (Fig. 6).

II. Cooperative Protection of LDH against Urea Denaturation Given by DPN and Substrates

(*A*) *Effect of DPN⁺+Pyruvate*—The effect of the concentration of pyruvate in the presence of 10^{-3} *M* DPN^+ against urea denaturation of LDH is illustrated in Fig. 4 and the cooperative protection of LDH by the couple was given markedly in the concentration of 10^{-2} *M* pyruvate. The above experiment indicates that a higher concentration of

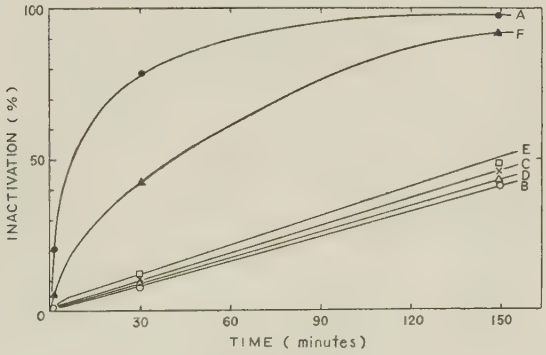


FIG. 5. Protective effect of DPNH and lactate on inactivation of lactic dehydrogenase in solution of urea.

Condition of treatment was the same as in Fig. 4. Curve A, control (without DPNH and lactate); Curve B, $+10^{-4}$ *M* DPNH; Curve C, $+10^{-4}$ *M* DPNH$+10^{-4}$ *M* lactate; Curve D, $+10^{-4}$ *M* DPNH$+10^{-3}$ *M* lactate; Curve E, $+10^{-4}$ *M* DPNH$+10^{-2}$ *M* lactate; Curve F, $+10^{-5}$ *M* DPNH.

pyruvate of about ten times as much as DPN^+ is required for the protection.

(*B*) *Effect of DPNH+Lactate*—The cooperative protection against urea denaturation was hardly observed in a couple of *DPNH+Lactate* (Fig. 5).

(*C*) *Effect of DPN⁺+Lactate*—In order to determine the quantitative relation of the couples on the protection, combinations of the couple mentioned above consist of non-reactive coenzyme and substrate, because the combination of reactive ones, *i.e.*, *DPNH+pyruvate* or *DPN⁺*

+lactate, is able to advance enzymatic action and two types of coenzyme and two substrates in a test solution present in different portions at each times. Under the present experimental condition at pH 7.6, however, the equilibrium of enzymatic action was in favor of lactic acid formation, so that it was possible to study the effect of the combination of *DPN*$^+$*+Lactate*. Fig. 6 illustrates the result, from which a small cooperative protection is observed in this couple.

III. Protective Effect of Some DPN-Related Compounds

The results mentioned above strongly point to the concept that

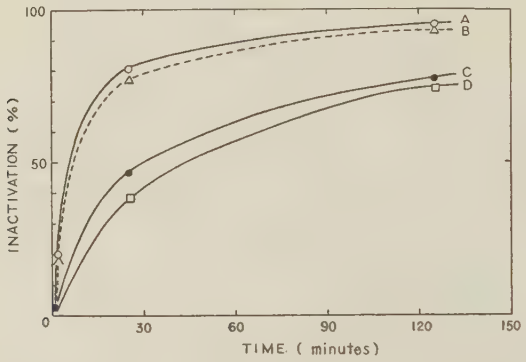


FIG. 6. Effect of DPN$^+$ and lactate on inactivation of lactic dehydrogenase in solution of urea.

Condition of treatment was the same as in Fig. 4. Curve A, control; Curve B, $+10^{-2}$ *M* lactate; Curve C, $+10^{-3}$ *M* DPN$^+$; Curve D, $+10^{-3}$ *M* DPN$^+$ $+10^{-2}$ *M* lactate.

a DPN-LDH complex is very stable in the urea solution, but a substrate-LDH is not, and this seems to be caused by the difference in the mode of combination. The protective effect given by some DPN-related compounds, such as ATP, AMP, adenine, and pyrophosphate, was examined in order to obtain some suggestion on the mode of binding of DPN with LDH, and ATP was the only effective compound. The protection given by ATP, however, was smaller than that of equimolar DPN$^+$ and had no cooperative protection in a couple with pyruvate, as illustrated in Fig. 7, from which the qualitative difference between the mode of DPN$^+$ and ATP was indicated.

IV. Protection of ADH from Urea Denaturation

The protection of ADH from urea denaturation was also observed in a similar way to those described above, but acetaldehyde was omitted from the reaction system because this reagent acted as an accelerator of urea denaturation. Fig. 8 illustrates the results which shows that the protection also appeared strongly by the addition of DPN^+ and DPNH while ethyl alcohol, one of the substrate, had only a slight effect as in the case of LDH. However, the fact that DPN^+ had a slightly stronger effect than DPNH was different from the results obtained with LDH. The cooperative effect in a couple of *DPN^++ethyl alcohol* was

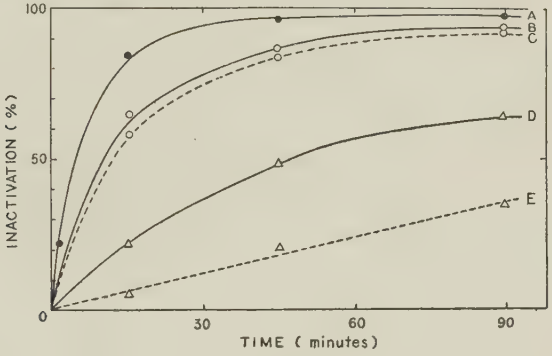


FIG. 7. Effect of ATP in comparson with DPN^+ on inactivation of lactic dehydrogenase in solution of urea.

Condition of treatment was the same as in Fig. 4. Curve A, control ; Curve B, $+5\times10^{-3}$ *M* ATP ; Curve C, $+5\times10^{-3}$ *M* ATP+ 10^{-2} *M* pyruvate ; Curve D, $+5\times10^{-3}$ *M* DPN^+ ; Curve E, $+5\times10^{-3}$ *M* $DPN^+ + 10^{-2}$ M pyruvate.

also observed in this case, but a couple of *DPN^++pyruvate* was without effect. These results show the important role of the complex of DPN and specific substance with dehydrogenase in stabilization of intramolecular structure of any DPN-linked dehydrogenase protein.

V. Effect of DPN on Heat Denaturation of ADH and LDH

LDH solution at pH 7.1 was very stable at room temperature, but about 96 per cent of initial activity was lost by heating at 60° for 10 minutes. In the presence of 1.5×10^{-4} *M* DPN^+ or DPNH, this decrease in enzymatic activity was 88 or 42 per cent, respectively. From the above results, it was also confirmed in heat denaturation of LDH that protec-

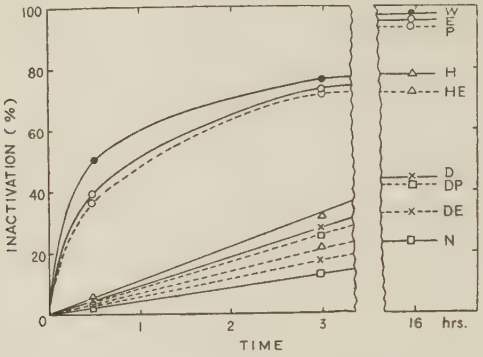


FIG. 8. Effect of DPN^+, DPNH, ethyl alcohol, pyruvate, and their combinations on inactivation of crystalline alcohol dehydrogenase from yeast in the presence of urea.

Condition of treatment was 3 *M* urea, at pH 7.0 and at 30°. W, control ; E, $+1.5\times10^{-3}$ *M* ethyl alcohol ; P, $+1.5\times10^{-3}$ *M* pyruvate ; H, $+1.5\times10^{-3}$ *M* DPNH ; D, $+1.5\times10^{-3}$ *M* DPN^+ ; HE, $+1.5\times10^{-3}$ *M* DPNH$+1.5\times10^{-3}$ *M* ethyl alcohol ; DE, $+1.5\times10^{-3}$ *M* $DPN^+ + 1.5\times10^{-3}$ *M* ethyl alcohol ; DP, $+1.5\times10^{-3}$ *M* $DPN^+ + 1.5\times10^{-3}$ *M* pyruvate ; N, no urea.

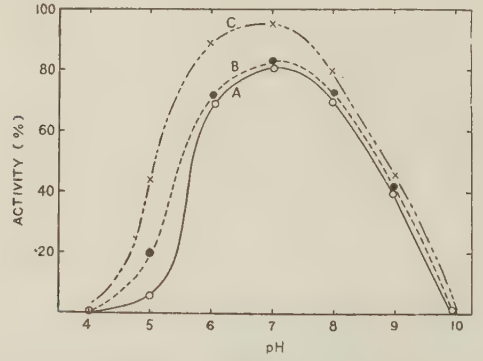


FIG. 9. Effect of DPN^+ and DPNH on heat denaturation of LDH at various pH.

1.5×10^{-6} *M* LDH was treated with heating at 55° for 60 minutes in the presence of 1.5×10^{-4} *M* DPN^+ or DPNH and the remaining activity was measured. The full activity was measured at pH 7.1 and 25°. The buffers at each pH used in this experiment were prepared by the addition of conc. NaOH into the mixture of equal volume of 0.5 *M* acetic acid, boric acid, and NaH_2PO_4 which was at pH 4.0. Curve A, no addition ; Curve B, $+1.5\times10^{-4}$ *M* DPN^+ ; Curve C, $+1.5\times10^{-4}$ *M* DPNH.

tive effect given by the presence of DPN^+ or DPNH and the effect given by DPNH is larger than the one by DPN^+. As illustrated in Fig. 9, the protective effect of DPN^+ and DPNH is observed against heat denaturation of LDH at 55° between pH 5.5 and 9.0 and the ratio of protection was better in acidic site. A similar effect of DPN mentioned above was also observed in the case of ADH.

DISCUSSION

Since the existence of protective effect of DPN against urea or heat denaturation of LDH was also confirmed by the above studies as in the case of GDH reported in the previous paper (*6*), it was demonstrated that the formation of DPN-dehydrogenase complex is highly effective in stabilizing the DPN-linked dehydrogenase proteins. From the Results *I*, the efficiencies of protection against urea denaturation of LDH will be in the following order:

$$DNPH > DPN^+ \gg \text{pyruvate} > \text{lactate}$$

Since the equilibrium of LDH under the present experimental condition at pH 7.6 is in favor of the formation of DPN^+ and lactic acid, this order of protection seems to be dependent upon the affinity of these substances to LDH. However, the protection given by DPN^+ was larger than the one given by DPNH in urea denaturation of ADH as described in Results *IV*. The difference between the protection given by DPN^+ and by DPNH against denaturation can not be explained sufficiently by the relation of Michaelis constants which were calculated in ADH by Racker (*8*) and in LDH by Meister (*16*), Neilands (*17*), and Schwert and Hakala (*18*) for these substances. Though it is thought that the formation of the complex of these substances and dehydrogenases is closely connected with their mutual affinity, the above results indicate the important roles of the mode of complex formation against the stabilization of dehydrogenase proteins.

According to Racker and Krimsky (*19*), Velick and Hayes (*20*), and Segel and Boyer (*21*), the reactive SH groups of DPN-linked dehydrogenase bind with pyridine ring of DPN, and according to Theorell *et al.* (*22*) other groups of enzyme and DPN are reactive. Since the data in Results *III* shows the existence of protection by ATP against urea denaturation of LDH, a part of adenosine or pyrophosphate in the molecule of DPN may also play an important role in the formation of DPN-enzyme complex.

On the other hand, the protective effect was hardly given by the substrates against enzyme activity (see Results *I*, *II* and *IV*). These results lead us to the conclusion that the stabilization of dehydrogenase by some substance needs the combination to be present extensively on the surface of enzyme proteins.

The existence of cooperative protection given by a couple of DPN and specific substrates may also be explained by this conclusion, because some kind of mutual relation between the specific substrate and DPN on the surface of dehydrogenase is considered as a part of the course of mechanism of enzymatic action and the cooperative protection is not given by the couple of ATP and pyruvate (see Results *II* and *III*). Based on thermodynamical studies, Soquet and Laidler (*23*) reported the reduction of entropy in the process of dehydrogenation of lactic acid by heart muscle LDH and DPN. Since the present result is concerned with the stabilization of dehydrogenase bound with DPN and substrate under the conditions promoting the denaturation, it is difficult to connect directly our result with theirs. However, the effect of the change in the intramolecular structure of DPN-linked dehydrogenase given by DPN and substrates must be considered in the study of reaction mechanism of DPN-linked dehydrogenase. In connection with this respect, it is of interest to note that, in the case of living cells, it will be expected from the above results that a more effective stabilization of the enzyme will be given by some large molecular substances such as nucleic acid or phospholipide.

In heat denaturation of LDH and ADH, the protective effect of DPN is observed, so that the fact will be available in the purification of DPN-linked dehydrogenases as in the case of other enzymes in which enzymes were purified by heating in the presence of the substrate. On the other hand, the fact that the protection given by DPN is better in acidic site will give some suggestion on the binding of DPN with dehydrogenase.

SUMMARY

Extensive studies were carried out in an attempt to clarify whether the two DPN-linked dehydrogenases, lactic and alcohol dehydrogenases, are stabilized in the presences of substrates, DPN, and their related compounds. Using the bacterial proteinase method for the determination of the ratio of denaturation and the amperometric titration method

for the measurement of the number of reactive SH-groups in enzyme protein, it was demonstrated that the inactivation and denaturation of LDH in a solution of urea proceeded in parallel. Under this condition, both dehydrogenases were considerably protected by DPN^+ and DPNH, and slightly by some substrates. When some of the substrates and DPN were coupled, the protection against urea denaturation was markedly enhanced. The combination of DPN^+ and pyruvate protected LDH from the denaturation markedly, but not ADH. The latter enzyme, on the other hand, was protected by the combination of DPN^+ and ethyl alcohol. While ATP also protected LDH at a higher concentration from the action mentioned above, the protection was not increased by the addition of pyruvate.

The stabilizing effect of DPN^+ and DPNH on both enzymes was also essentially confirmed in the case of heat denaturation.

Based on the above results, the mechanism of the stabilization of DPN-linked dehydrogenases protein was discussed.

REFERENCES

(*1*) Okunuki, K., Hagihara, B., Matsubara, H., and Nakayama, T., *J. Biochem. (Japan)*, **43**, 453 (1956)

(*2*) Hagihara, B., Nakayama, T., Matsubara, H., and Okunuki, K., *ibid.*, **43**, 469 (1956)

(*3*) Hagihara, B., Nakayama, T., Matsubara, H., and Okunuki, K., *ibid.*, **43**, 483 (1956)

(*4*) Hagihara, B., Shibata, T., Sekuzu, I., Hattori, F., Nakayama, T., Nozaki, M., Matsubara, H., and Okunuki, K., *ibid.*, **43**, 495 (1956)

(*5*) Sekuzu, I., Hagihara, B., Hattori, F., Shibata, T., Nozaki, M., and Okunuki, K., *ibid.*, **44**, 587 (1957)

(*6*) Nozaki, M., Sekuzu, I., Hagihara, B., Yonetani, T., Nakai, M., and Okunuki, K., *ibid.*, **44**, 595 (1957)

(*7*) Straub, F. B., *Biochem. J. (London)*, **34**, 483 (1940)

(*8*) Racker, E., *J. Biol. Chem.*, **184**, 313 (1950)

(*9*) Okunuki, K., Hagihara, B., Sekuzu, I., Nozaki, M., and Nakai, M., *J. Biochem. (Japan)*, **42**, 389 (1955)

(*10*) Dubois, K. P., Albaum, H. G., and Potter, V. R., *J. Biol. Chem.*, **147**, 699 (1943)

(*11*) Clift, F. P., and Cook, R. D., *Biochem. J. (London)*, **26**, 1788 (1932)

(*12*) Kolthoff, I. M., Stricks, W., and Morren, L., *Anal. Chem.*, **26**, 366 (1954)

(*13*) Ingram, V. M., *Biochem. J. (London)*, **59**, 653 (1955)

(*14*) Kubo, H., *Symposia on Enzyme Chemistry (Japan)*, **5**, 1 (1950) ; Iwatsubo, M.,

Ishibashi, M., Takashina, T., Ibaraki, K., and Kashida, H., *ibid.*, **10**, 135 (1954)

(*15*) Burton, K., *Biochem. J.* (*London*), **48**, 458 (1951)

(*16*) Meister, A., *J. Biol. Chem.*, **184**, 117 (1950)

(*17*) Neilands, J. S., *J. Biol. Chem.*, **199**, 373 (1952)

(*18*) Schwert, G. W., and Hakala, M. T., *Arch. Biochem. Biophys.*, **38**, 55 (1952)

(*19*) Racker, E., and Krimsky, I., *J. Biol. Chem.*, **198**, 731 (1953)

(*20*) Velick, S. F., and Hayes, J. E., *J. Biol. Chem.*, **203**, 545 (1953)

(*21*) Segel, H. L., and Boyer, P. D., *J. Biol. Chem.*, **204**, 265 (1953)

(*22*) Theorell, H.,, Nygaard, A. P., and Bonnichsen, R., *Acta Chem. Scand.*, **9**, 1148 (1955)

(*23*) Soquet, I. M., and Laidler, K. T., *Arch. Biochem.*, **25**, 171 (1950)

The Journal of Biochemistry, Vol. 44, No. 9, 1957

A SOLUBLE, AUTOXIDIZABLE AND CO-BINDING PIGMENT FROM A STRAIN OF HALOTOLERANT BACTERIA

Sirs:

Sato has observed a CO-binding pigment in a suspension of a strain of halotolerant bacteria (*1*). Recently we could extract a CO-binding substance from the same organism. The purpose of this note is to outline the results of purification and some properties of the pigment, although the identity of these two CO-binding pigments is not yet certain.

A halotolerant bacterium strain 203 was grown aerobically in a peptone-broth medium containing 10 per cent NaCl, 1 per cent KNO_3, and 0.02 per cent $MgSO_4 \cdot 7H_2O$, pH 7.2, at 34° for 24 hours. Cells were harvested with a Sharples supercentrifuge, washed once with 10 per cent NaCl solution and lyophilized. The suspension of the lyophilized cells was dialysed against 0.01 *M* phosphate buffer, pH 6.8, overnight and centrifuged to obtain the supernatant solution (Crude extract). The precipitate obtained between 40 and 50 volume per cent acetone (Fraction A) was adsorbed on and eluted from calcium phosphate gel with 0.1 *M* phosphate buffer, pH 6.8. The eluate (Fraction G) was dialyzed against distilled water with stirring for several hours. It was chromatographed on the chloride form of Dowex 2 equilibrated with 0.02 *M* Tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.3, and eluted with 1 *M* Tris buffer, pH 7.3 (Fraction R), yielding a yellow solution. Fraction R was not yet homogeneous in analytical ultracentrifuge.

On spectrophotometric observation of fraction R, it was shown to have the characteristic absorption spectra of cytochromes (Fig. 1). The peaks were found at 405 mμ and 635 mμ in the oxidized form and at 420 mμ with a shoulder about 435 mμ, 521 mμ and 554 mμ with a shoulder around 548 mμ in the reduced one. These peaks were clearly distinguishable from those of cytochrome b_4 (*2*).

This pigment was shown to be autoxidizable. In spectrophotometric experiments using Thunberg tube-type cuvettes, the reduced Soret band shifted to the oxidized form upon introduction of air (Fig.

2). The rate measured by the decrease of optical density at 420 mμ was accelerated about 3-fold specifically by the addition of 5×10^{-4} *M* $MnCl_2$ (Fig. 2). Autoxidation of this pigment led us to examine the distribution pattern of the cytochrome oxidase which had already been reported as a halophilic enzyme from the same organism (*3*). In this

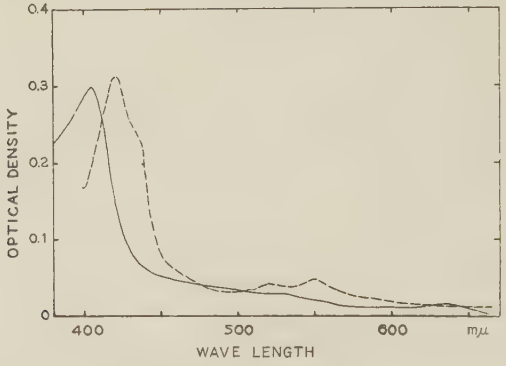


FIG. 1. Absorption spectra of the pigment.
Full line : oxidized. Dotted line : reduced.

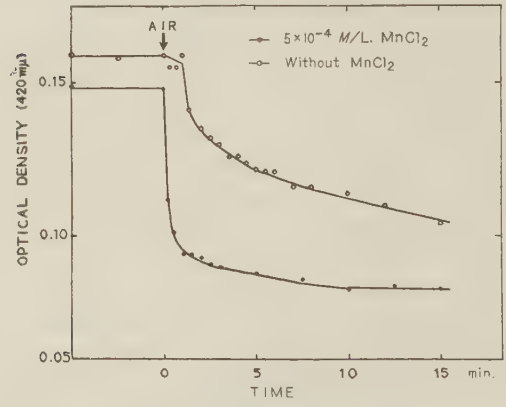


FIG. 2. Autoxidation and effect of Mn++.

case activity was measured by the *p*-phenylenediamine technique using Warburg manometers (*3*). A suspension of lyophilized cells, the 105,000 $\times g$. supernatant and the residue were tested in the presence of 1 *M* NaCl. About 97 per cent of the *p*-phenylenediamine oxidase ac-

tivity of the suspension were found in the residue and only 3 per cent in the supernatant, while almost all the pigment was obtained in the supernatant. This shows that the cytochrome oxidase and the autoxidizable pigment are not identical.

When the reduced pigment was incubated with 1 atm. of CO in the dark, marked change of absorption spectra occurred. The Soret band shifted to shorter wave length side (416–417 mμ) and illumination restored the change completely (Fig. 3). This fact is quite interesting, since, as described below, this preparation contains CO-sensitive hydroxylamine reductase activity (*4*).

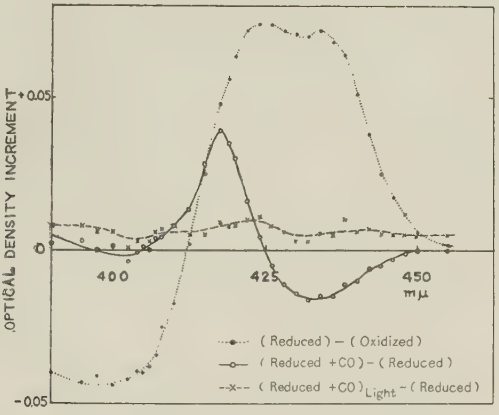


FIG. 3. CO-binding spectrum and photodissociation. (Difference spectra)

Besides autoxidizability and CO-binding ability, the pigment has a strong hydroxylamine reductase activity measured by the reduced methylene blue technique as already described elsewhere (*4*). There is a remarkable parallelism between the reductase activity and the autoxidizability, thus suggesting that these two activities are the attribute of one single protein. The more detailed investigation has been carried on about the nature of the present pigment as well as that of the reductase and will be presented elsewhere.

REFERENCES

(*1*) Sato, R., Unpublished observation, cited by B. Chance in "The Physical Chemistry of Enzymes." Discussions of the Faraday Society, 1955, No. 20, p. 205

(2) Egami, F., Itahashi, M., Sato, R., and Mori, T., *J. Biochem.*, **40**, 527 (1953)
(3) Yamada, T., and Asano, A., *Comp. rend. Soc. Biol.*, **168**, 1310 (1954)
(4) Taniguchi, S., Mitsui, H., Nakamura, K., and Egami, F., *Ann. Acad. Sci. Fennicae, Ser. A, II* (Biochemistry of Nitrogen), **60**, 200 (1955) ; Taniguchi, S., Sato, R., and Egami, F., Inorganic Nitrogen Metabolism, edited by W. D. McElroy and B. Glass, the Johns Hopkins Press, Baltimore, p. 87 (1956)

ADDENDUM

The pyridine hemochromogen was prepared by alkaline treatment in the presence of pyridine. The absorption maxima of the reduced hemochromogen coincided with those of mammalian cytochrome c. It is concluded that the present pigment is a hitherto unknown new cytochrome and is one of c type cytochromes. We should like to name it cytochrome c^{554} (Halotolerant Coccus 203).

Department of Chemistry,
Faculty of Science,
Nagoya University,
Nagoya

MASAKIYO KONO
SHIGEHIKO TANIGUCHI
FUJIO EGAMI

(Received for publication, July 9, 1957)

The Journal of Biochemistry, Vol. 44, No. 9, 1957

PROVISIONAL PROPOSALS FOR CLASSIFICATION AND NOMENCLATURE OF CYTOCHROMES

Rapid increase in recent years in the number of cytochromes found in the biological kingdom seems to have caused some confusions in the system of their classification and nomenclature. The purpose of this communication is to make some provisional proposals for these problems.

As the present proposals intend only to suggest a rational set of rules for the classification and nomenclature of cytochromes, we are not in the situation to discuss problems arising from the apparent uncertainty inherent to the definition of this group of enzymes. Nevertheless, it seems desirable to make clear, before entering into the description of proposals, what we would like to call cytochromes in this communication. In our views, cytochromes could best be defined as those intracellular hemoproteins, the normal functions of which appear to consist in the transfer of electrons or other respiratorical actions in the biological oxidation. Electrons are transferred in these cases by virtue of the ability of the heme-bound iron atoms to undergo reversible oxidation and reduction between ferric and ferrous states. The term hemoprotein is used here to imply not only typical iron-porphyrin proteins but also those conjugated proteins whose prosthetic groups are iron chelates of chlorins and other compounds structurally related to porphyrins.

According to the definition given above, some of the hemoproteins usually classified into peroxidases or even hemoglobins may have to be called cytochromes. Horse raddish peroxidase may be an example of such cases ; this hemoprotein is, besides being active as peroxidase, able to function as a dihydroxymaleic acid oxidase and in this function the heme moiety seems to undergo reversible oxidation and reduction. In fact, we are still almost ignorant as to what is the normal function of the enzyme. It should, however, be emphasized that, as already stated above, these complications are something beyond the scope of the following proposals which merely aim to give suggestions for the classification and nomenclature of those hemoproteins which are assigned to cytochromes by a majority of investigators.

PROPOSALS

(*A*) Although enzymes are usually classified according to the chemical nature of substrates or the types of reactions catalysed, it seems difficult to apply this principle to cytochromes. It is therefore proposed that the classification of cytochromes should be made on the basis of the types of hemes they contain regardless of their catalytic functions and of their physicochemical properties.

(*B*) At least four types of hemes have so far been known to occur in cytochromes and their structural principles fully or almost fully defined. These are (1) heme a, (2) protoheme, (3) heme c, and (4) so-called heme a_2. Corresponding to these hemes, four groups of cytochromes can at present be distinguished. It is our proposal that these four groups of cytochrmoes should be named as follows:

Proposed group name	Characteristic heme
Cytochrome a	Heme a
Cytochrome b	Protoheme
Cytochrome c	Heme c
Cytochrome a′	So-called heme a_2

It should be noted here the terms cytochromes a, b, c, and a′ are used in the above list to denote respective groups and not individual cytochromes. The name cytochrome a′ is newly adopted to represent a group of cytochromes characterized by heme a_2 which has been isolated from bacteria containing " cytochrome a_2 " and its chemical structure shown to differ from that of heme a.

(*C*) According to the above rule, a cytochrome could legally be assigned to a particular group only when its heme moiety has been isolated and identified by organic chemical procedures. Such identification of cytochrome-hemes is, however, by no means an easy task and so far has been conducted with only a limited number of cytochromes. A more convenient method by which the assignment can be made is, therefore, desired. It is proposed that such preliminary assignment should be done on the basis of the position of α-band in the absorption spectrum due to the pyridine hemochromogen of the cytochrome in question, since the absorption spectra of pyridine hemochromogens appear to reflect the types of hemes most precisely. The assignment could thus be made as follows:

Position of α-band of pyridine hemochromogen	Cytochrome group
about 585 mμ	a
„ 557 „	b
„ 550 „	c
„ 613 „	a′

(*D*) Although a number of cytochromes have not yet been characterized even by the spectroscopic observation of their pyridine hemochromogens, most of them have already been classified into types a, b or c solely based on the position of α-band of their reduced forms. For such cytochromes, it is advisable to preserve the group names already assigned until the absorption spectra of their pyridine hemochromogens have been determined. It is, however, recommended that the spectroscopic observation of pyridine henochromogen spectra should hereafter be done before a cytochrome is classified into the groups.

(*E*) In view of the present status of our knowledge of cytochromes, it seems quite difficult to further classify them into subgroups according to their properties other than the types of their hemes. In the present proposals, therefore, no rules for such subclassification are given.

(*F*) When it is necessary to distinguish individual cytochromes belonging to the same group, the distinction should be made by specifying the positions of α-bands in the absorption spectra of their reduced forms, their sources, and any of their properties such as their normal oxidation-reduction potentials. The simplest set of specifications should be selected at each occasion sufficient to permit the desired distinction. It is our proposal that, in so specifying, the position of α-band (wavelength in mμ) should be put at the upper right of the letter referring to the cytochrome group and the source and other properties inserted in a parenthesis placed after the letter. These formalities may be understandable by the following examples:

Cytochrome a^{605} ; Cytochrome b^{560} (*Escherichia coli*) ;
Cytochrome c (*Bacillus subtilis*) ;
Cytochrome c (Heart muscle, $E_o' = 0.26$ V) ; *etc.*

(*G*) As to those cytochromes which have been given numbered names such as cytochromes a_1, b_2, *etc.*, it is proposed that these numbers may be preserved. The number should, as is currently in use, be placed at the lower right of the letter. The followings are several examples of

the numbered cytochromes:

Cytochrome a_3 ; Cytochrome b_1^{560} ; Cytochrome c_1 (yeast) ;
Cytochrome c_3^{553} (*Desulfovibrio desulfuricans*) ;
Cytochrome a_2' ; *etc.*

(*H*) Some cytochromes such as cytochromes f and h are named by letters other than a, b, c, and a'. It is proposed that these cytochromes should be renamed according to the types of hemes they contain. Thus, cytochrome f could be assigned to the group c and cytochrome h probably to the group b. It is proposed that the letters such as f and h could be preserved as suffixes to the group names in order to avoid confusions. Cytochrome f may thus be expressed as cytochrome c_f, cytochrome c_f^{555} *etc.*, and cytochrome h as cytochrome b_h, *etc.*

Commission on Cytochromes,
Liaison Committee for Biochemical
Research, Science Council of Japan,
Ueno Park, Tokyo, Japan

FUJIO EGAMI
MAKOTO ISHIMOTO
TAKESHI MORI
YASUYUKI OGURA
KAZUO OKUNUKI
RYO SATO

(Received for publication, August 8, 1957)